m^6A modification in gastric cancer:

the function of the YTH family of proteins

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A todos os que,
pelo seu exemplo e capacidade de partilha, me têm ajudado a crescer.

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Agradecimentos

«Todo o trabalho criativo é, na essência, solitário, porque a disciplina e o método assim o exigem, contudo, o resultado final, é fruto de uma interioridade conjunta que motiva, une e congrega o esforço, o trabalho, a intuição e o talento de múltiplas pessoas que connosco aceitam assumir tão grande desafio. Nenhum acto criativo é por isso fruto de um Homem só.» (Abrunhosa, 1994)[1]

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Aos meus pais e ao meu irmão, pelo seu apoio total e incondicional, e por ajudarem a afastar as nuvens que tentavam obscurecer o meu caminho…

[1]*Abrunhosa, Pedro (1994). Agradecimentos. In Pedro Abrunhosa e Bandemónio, Viagens [CD áudio]. Porto. Polygram Discos, S. A.*

Resumo

O cancro gástrico (CG) é a quarta causa de mortalidade global por cancro, dependendo a sua evolução clínica e tratamento do seu estadio. Além da identificação de biomarcadores com valor clínico para uma atribuição bem-sucedida de pacientes a regimes de tratamento, é essencial a identificação de novos alvos tumorais.

A modificação de RNA m^{6}A é a mais prevalente em moléculas de mRNA em eucariotas, sendo tal modificação reversível e dependente de três tipos principais de enzimas, que depositam, removem e reconhecem as modificações no RNA. Ao último grupo, pertence a família de proteínas YTH, a qual partilha domínios YTH quase idênticos entre si, os quais reconhecem m^{6}A em moléculas de RNA. A YTHDF3, anteriormente demonstrada como a mais prevalente no CG, tem um impacto significativo na motilidade e sobrevivência celular. Ora, dada a falta de evidências sobre os efeitos específicos das proteínas YTHDF no mRNA modificado com m^{6}A em CG, o nosso objetivo foi entender o nível de sobreposição de função entre as três proteínas usando duas linhagens celulares de CG e mutantes ΔYTHDF3 como modelos.

Para tal, avaliámos a expressão de YTHDF1/2 em AGS e SNU638 e respetivos mutantes ΔYTHDF3, estudámos a expressão de YTHDF1/2 em tecidos normais e avaliamos o impacto de YTHDF1/2 em células do CG, regulando negativamente sua expressão pelo uso de siRNAs.

YTHDF1/2/3 mostraram estar presentes principalmente no citosol de tecidos normais, e os seus níveis de expressão permanecem idênticos em mutantes ΔYTHDF3 e não mutantes, sugerindo que não há mecanismo compensatório e que cada RBP tem funções algo redundantes com as demais. A sequenciação de mRNA apresentou MISP como um dos genes mais alterados em ΔYTHDF3, porém a regulação negativa das proteínas YTHDF teve efeitos contraditórios na expressão de MISP, dependendo do modelo celular utilizado, possivelmente devido à existência de diferentes alvos YTHDF3 em ambas as linhagens, o que pode implicar a expressão aberrante de diferentes genes-chave envolvidos na sobrevivência celular.

Os resultados aqui apresentados destacam a relevância das proteínas YTHDF, particularmente YTHDF3 como um possível fator na compreensão do papel que as modificações m^{6}A podem acarretar para o CG.

Palavras-chave: cancro gástrico, compensação, m^{6}A, metilação de RNA, MISP, redundância, YTHDF1, YTHDF2, YTHDF3

Abstract

Gastric cancer (GC) remains the fourth leading cause of global cancer mortality, with patient's clinical outcome and treatment strongly dependent on TNM staging, which does not consider tumour heterogeneity. Thus, identification of biomarkers with clinical value for the successful assignment of patients to treatment regimens and the identification of new tumour targets are clear unmet needs in GC.

m^{6}A RNA modification is the most prevalent chemical modification in mRNA molecules in eukaryotes. This modification is reversible, and dependent on three key enzyme types, which deposit, remove and recognise RNA modifications. The latter belong to the YTH family of proteins and share nearly identical YTH domains which recognise m^{6}A in a methylation-dependent manner. YTHDF3 previously shown to be the most prevalent in GC, has a significant impact in cell motility and survival. Yet, given the lack of evidence on the specific effects of the YTHDF proteins on the m^{6}A-modified mRNA in GC, our aim was to understand the level of function overlap between the three proteins using two gastric cancer cell lines and ΔYTHDF3 mutants as models.

To do so, we evaluated the expression of YTHDF1/2 in AGS and SNU638 and respective ΔYTHDF3 mutants, we studied the expression in YTHDF1/2 in normal tissues and assessed the impact of YTHDF1/2 in GC cells by downregulating their expression using siRNAs.

YTHDF1/2/3 were showed to be mostly present in the cytosol of normal tissues, and their expression levels remain identical in wild-type and ΔYTHDF3 mutants, suggesting that there is no compensatory mechanism and that each RBP has some level redundant functions. The mRNA-sequencing performed presented MISP as one of the most altered genes on ΔYTHDF3, yet the downregulation of YTHDF proteins had contradictory effects on MISP expression, depending on the cell line model used, possibly due to the existence of different YTHDF3 targets in both cell lines, which could entail different aberrant expression of key genes involved in cell survival.

The results presented in this work highlight the relevance of the YTHDF proteins, particularly YTHDF3 as a possible key factor in understanding what m^{6}A modifications may entail in GC.

Keywords: compensation, gastric cancer, m^{6}A, RNA methylation, MISP, redundancy, YTHDF1, YTHDF2, YTHDF3

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List of abbreviations

The following list describes relevant abbreviations and acronyms used.

3'UTR	Three prime untranslated region
5'UTR	Five prime untranslated region
AGS	Adenocarcinoma gastric cell line
ALKBH5	AlkB homolog 5, RNA demethylase
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CCR4-NOT	Carbon catabolite repression – negative on TATA-less
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
E-cadherin	Epithelial cadherin; CDH1
ECL	Enhanced chemiluminescence substrate
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FMRP	Fragile X mental retardation protein
FTO	Fat Mass and Obesity-Associated protein
GC	Gastric Cancer
HER2	Human epidermal growth factor receptor 2
HNRNP	Heterogeneous nuclear ribonucleoproteins
H. pylori	*Helicobacter pylori*
IHC	Immunohistochemistry
KH	K homology domain

KO	Knock-out
m^6A	N^6-Methyladenosine
MALT	Mucosa-associated lymphoid tissue
METTL3	Methyltransferase-like protein 3
METTL14	Methyltransferase-like protein 14
MISP	Mitotic Spindle Positioning
mRNA	messenger RNA
Na3VO4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
PB	Presto Blue
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl fluoride
PUD	Peptic ulcer disease
RBDs	RNA Binding Domains
RGG	Arginine/glycine -rich domain
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
sgRNA	Single guide RNA

siRNA	Small interfering RNA
SNU638	Seoul National University 638 cell line
SNP	Single nucleotide polymorphisms
TBS-T	Tris-buffered Saline Supplemented with Tween20
TMAs	Tissue Microarrays
Tris	Tris(hydroxymethyl)aminomethane
tRNAs	Transfer RNAs
VIRMA	Vir like m^{6}A methyltransferase associated
WHO	World health organization
WTAP	Wilms tumour 1 associated protein
YTH-domain	YT521-B homology domain
YTHDC 1-2	YTH domain containing 1 -2
YTHDF	YTH domain family of proteins
YTHDF1	YTH domain-containing family protein 1
YTHDF2	YTH domain-containing family protein 2
YTHDF3	YTH domain-containing family protein 3

1– Introduction

1.1 – Gastric Cancer

Gastric cancer (GC) is the fifth most common cause of cancer, being also the fourth cause of cancer related deaths worldwide, with 769,000 annual casualties (Sung *et al.,* 2021). GC has a higher incidence in countries with a high human developmental index, with about 74% of the world cases occurring in Asia (Figure 1), mainly in China (Ferlay *et al.,* 2021; Sung *et al.,* 2021).

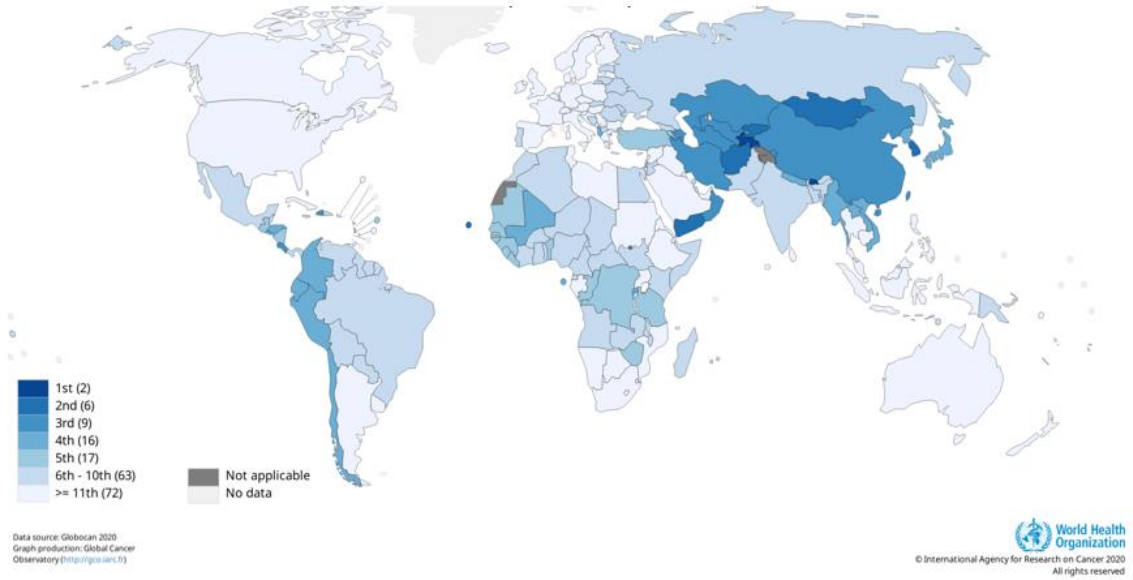


Figure 1 – Cancer statistics from 2020. Estimated stomach cancer age-standardized incidence rates world map. All statistics are from 2020 for both sexes and all ages. Adapted from: Global Cancer Observatory (Ferlay *et al.,* 2021; Sung *et al.,* 2021)

Nearly 90% of all GCs are adenocarcinomas (tumours in which the cancerous cells are arranged in the form of glands), arising either from cardia or non-cardia parts of the stomach. The remaining 10% of cancers developing from the stomach include mucosa-associated lymphoid tissue (MALT) lymphomas and leiomyosarcomas, which originate, respectively, from lymphoid tissue of the stomach and muscles surrounding the mucosa (Karimi *et al.,* 2014).

The stomach is divided into distinct regions (Figure 2), namely the cardia (the top part of the stomach), fundus, body, pylorus and the antrum, with the differences between these areas being due to their anatomy, histology, or both.

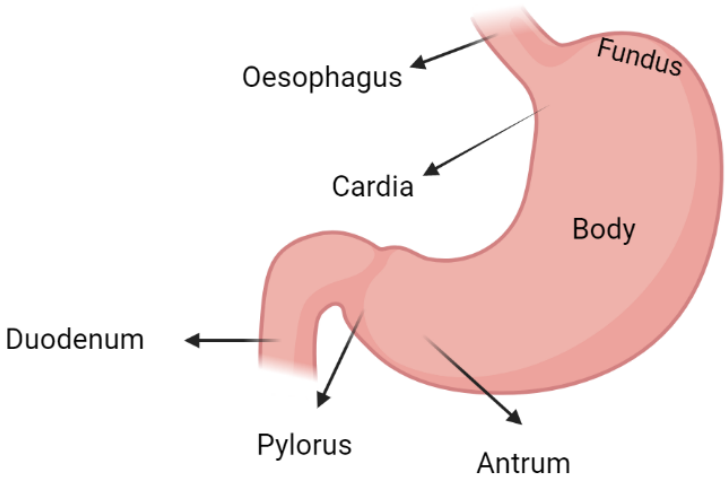


Figure 2 – Stomach anatomy.

Thus, with regard to GC, it is, traditionally, subdivided into two categories based on topography and histological features, cardia and non-cardia GC. Cardia GC refers to cancer found near the gastroesophageal junction, while non-cardia GC involves all the cancers located in the lower portion of the stomach (Karimi *et al.,* 2014).

There are a variety of factors that can contribute to the occurrence of GC, namely acquired mutations, single nucleotide polymorphisms (SNPs), chromosomal and microsatellite instability, somatic gene mutations and epigenetic alterations (Karimi *et al., 2014*). However, favourable environmental conditions are also connected to the development of GC, particularly infection by *Helicobacter pylori* (*H. pylori*) or Epstein–Barr virus (EBV), diet, smoking and hyper/hypogastrinaemia (Skierucha *et al.,* 2016). Common risk factors for both cardia and non-cardia GC include age, sex, radiation exposure, family history, smoking and abusive alcohol consumption (La Vecchia *et al.,* 1992; Lagergren *et al.,* 2000; Ladeiras-Lopes *et al.,* 2008).

At a global scale, non-cardia GC registers as the most frequent subtype of GC, and this is due to *H. pylori* infection and dietary factors (low fruit intake, high consumption of processed meat and grilled foods, or foods preserved by salting) (Blaser *et al.,* 2004; World Cancer Research Fund, 2018), which makes it more common in countries with a

low human developmental index. As a result of improved hygiene, food conservation and quality, and by controlling *H. pylor*i infection (Etemadi *et al.,* 2020), non-cardia GC is declining in developed countries, , whereas cardia GC is becoming more frequent (World Cancer Research Fund, 2018), especially since cardia GC relates to obesity and gastroesophageal reflux disease (Vaughan *et al.,*1995; Rubenstein *et al.,* 2010).

However, according to the World Health Organization (WHO), GC could also be classified according to its onset (Bosman *et al.,* 2010). Here, GC is divided into Early-Onset Gastric Cancer, when it occurs in patients with 45 years old or younger, and Conventional Gastric Cancers, which includes all the remaining groups of GC from Hereditary Diffuse Gastric Cancer, Sporadic Gastric Cancer and Gastric Stump Cancer. The majority of GC are Sporadic Gastric Cancers, that occur sporadically, and mainly affect people over 45 years of age (Figure 3). Generally, they are caused by a coincidence of many environmental factors (consider the factors mentioned above for the non-cardia GC). The second most common is Early-Onset Gastric Cancer, in which genetic factors seem to play a crucial role in its occurrence (Skierucha, *et al.,*2016). Gastric Stump Cancer is a particular case of GC, as it is defined by a carcinoma occurring in the gastric remnant after partial gastric resection for peptic ulcer disease (PUD) (Costa-Pinho *et al.,* 2013) representing 1.1-7% of all GC. Hereditary Diffuse Gastric Cancer is the least common GC, and is the result of inherited syndromes, most of which are autosomal dominant conditions leading to diffuse, poorly differentiated GC, that infiltrates into the stomach wall and causes thickening of the wall without creating a distinct mass (Sitarz *et al.,* 2018).

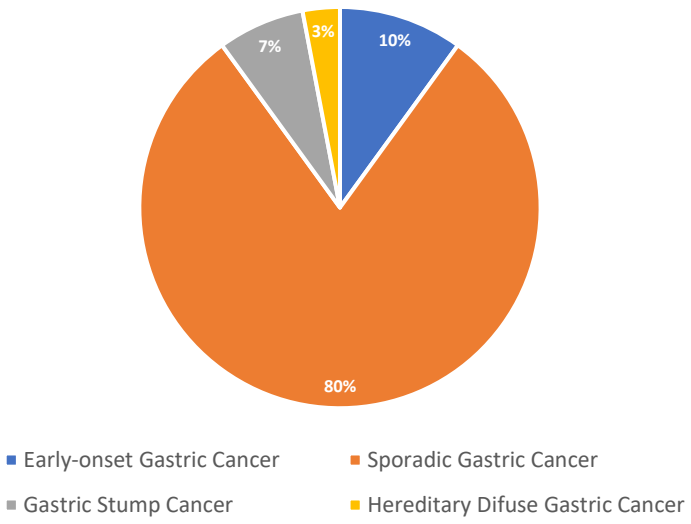


Figure 3 – Onsets of GC development. Adapted from: Machalowska *et al.,* 2020

As previously referred, there are a variety of acquired mutations that play an important role in GC. According to Oliveira, *et al*., 2006, approximately 30-40% of the Hereditary Diffuse Gastric Cancers are caused by E-cadherin (CDH1) mutations, a vital protein in cell-cell adhesion. Other mutations related to Hereditary Diffuse Gastric Cancers are the TP53 mutation in Li-Fraumeni syndrome, the STK11 mutation in Peutz-Jeghers syndrome, the APC mutation and the BRCA2 mutation (Skierucha *et al.,* 2016).

When considering Sporadic Gastric Cancer, various factors can precipitate the start of the malignant process, such as environmental factors, SNPs and various acquired mutations that lead to chromosomal instability, microsatellite instability, somatic gene mutations and epigenetic alterations. Collins, *et al*. 1998, suggests that SNPs are responsible for 90% of the human genetic variability. Many of these SNPs are GC related and can increase the predisposition to this type of cancer (Table 1). Some examples are SNPs in inflammatory response genes like IL1, IL17, TNFα and TLRs, in genes related with DNA repair after *H. pylori* infection like XPA, XPC and ERCC2, in genes related to metabolic pathways and in other genes like MUC1 and CDH1. Though SNPs only predispose to cancer, without initiating it, acquired mutations are, more frequently than not, responsible for the initiation of malignant processes. From these, the most frequent are somatic mutations that occur in genes such as TP53, CDH1, SMAD4, PIK3CA, RHOA, ARID1A, KRAS, MUC3, APC, ERBB1, PTEN, HLAB and B2M (Skierucha *et al.,*

2016). Epigenetic modifications also play an important role in malignant processes initiation, by modifying the nucleic acid structure.

Table 1 – Sporadic gastric cancer factors (Adapted from Skierucha *et al.,* 2016)

Factors	Examples related to SGC
SNPs	*IL1, IL17, TNF*α*, TLRs* (inflammatory response) *MUC1* (protection against invaders) *CDH1* (cell-to-cell adhesion) *XPA, XPC, ERCC2* (repair of DNA damage related to *H. pylori* infection) *MTHFR* (metabolism of foliate) *GSTT1, SULT1A1, NAT2, EPHX1* (metabolism of polycyclic aromatic hydrocarbons) *CYP2E1* (metabolism of xenobiotics) *PSCA*
Chromosomal Instability	Gain of copy number at 8q, 17q, 12q, 13q and 20q Amplification of *EGF* and *c-ErbB2* Amplification of *FGFR* Amplification of *ERBB2* Overexpression of *HGF* and *c-myc* *SLC1A2-CD44* fusion *ROS1* rearrangement
Loss of Heterozygosity	*APC*, *TP53*, *NME1*
Microsatellite Instability	*BAX, BCL10, FAS, CASPASE5, APAF1* (apoptosis related genes) *hMSH6, hMSH3, MED1, RAD50, BLM, ATR, MRE11* (DNA repair genes)
Somatic Gene Mutations	*TP53*, *CDH1*, *SMAD4*, *PIK3CA*, *RHOA*, *ARID1A*, *KRAS*, *MUC3*, *APC*, *ERBB1*, *PTEN*, *HLAB*, *B2M*, *FAT4*
Epigenetic Alterations	CpG island methylation of the promoters of *CDH1*, *CDKN2A*, *CDKN2B* and *hMLH1* miRNA variations
Environment	Diet *H. pylori* infection EBV infection Hyper/Hypogastrinaemia Smoking
Others	*COX2* overexpression

When the therapeutic approach is considered, a multidisciplinary team ought to be comprised of at least a surgeon, pathologist, gastroenterologist, medical and radiation oncologists (Kamiya *et al.,* 2018). The therapeutic approach is normally determined by the stage of the disease at the time of diagnosis, intention of therapy, patient's performance status and the technical possibilities available (Figure 4) (Sitarz *et al.,* 2018).

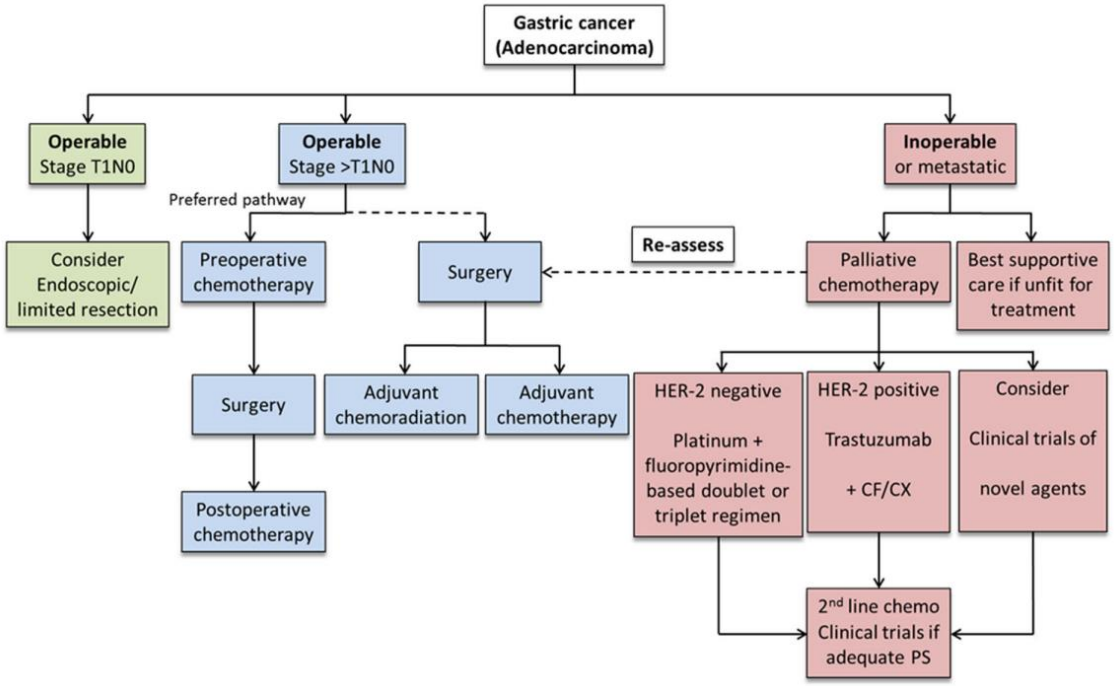


Figure 4 – Algorithm for the management of gastric cancer in Europe. From: Kamiya *et al*., 2018

To this date, surgery remains the main treatment for stages I to III, though a combined treatment with peri-operative chemotherapy could be recommended, particularly for the latter. For stage IV tumours, chemotherapy is the main treatment, here combined with surgery, whenever possible. Anti-human epidermal growth factor receptor-type 2 (HER2)-antibody is administered when the tumours express HER2, representing the only targeted therapy for GC available at the moment (Sitarz *et al.,* 2018).

At present, there is a clear need for the implementation of new treatment strategies for GC patients, which are currently underdeveloped, adding to very heterogeneous patient outcomes, even within the same stage.

Even though a steady decline in incidence and mortality has been observed over the last decades (Ferlay *et al.,* 2015; Ferro *et al.,* 2015; Sung *et al.,* 2021), thus far, GC is one of the most diagnosed cancer types and the mortality remains high, with a disturbing 5-year

survival rate of less than 35% (Sung *et al.,* 2021). As mentioned above, currently, one needs to consider cancer stage at the time of diagnosis in order to come up with a therapeutic approach. However, this approach does not consider tumour heterogeneity, which is a key variable to contemplate when deciding on the therapy plan. Therefore, GC treatments often fail, and new prognostic biomarkers are urgently needed to distinguish different tumour biological behaviours, namely, chemotherapy response, regardless of tumour extension or tumour type (Röcken & Behrens, 2015).

1.2 – Epitranscriptome

Whereas DNA epigenetic modifications are well-known to play a pivotal role in gene expression regulation, with its dysregulation being a consistent feature in multiple cancers (Akalin *et al.,* 2012), over the last few years, modern technologies have shed a new light on RNA biology and how it is more important and complex than it was previously considered. The new knowledge that RNA modifications, collectively referred to as the "epitranscriptome", were also involved in an additional layer of gene expression regulation (Peer *et al.,* 2017), contributing to its diversity of functions, and has led to a re-evaluation of the role of the RNA in health and diseases (Zaccara *et al.,* 2019; Destefanis *et al.*, 2021).

To this date, more than 150 distinct chemical modifications (Figure 5) have been identified (Boccaletto *et al*., 2022), making it clear that RNA transcripts are not merely transient copies of DNA and, similarly to what occurs with chromatin, they have the potential to regulate many gene functions, changing our view of the central dogma of biology. These modifications can affect different classes of RNAs, from messenger RNAs (mRNAs), ribosomal RNAs (rRNAs) to transfer RNAs (tRNAs).

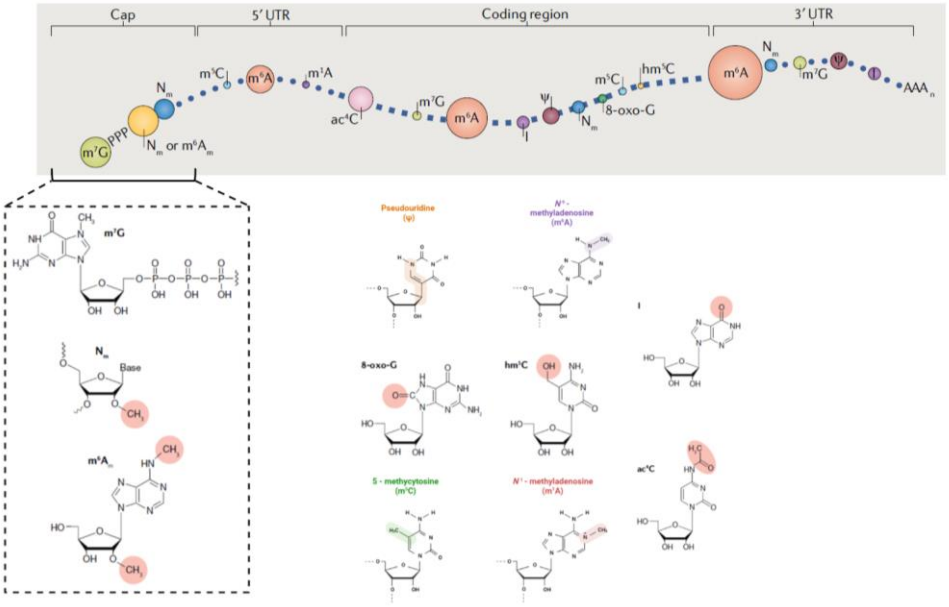


Figure 5 – Some examples of RNA modifications described on mRNAs and its locations. The proportions of the circles are a schematic representation of the abundance of each modification within the transcript. Adapted from: Zaccara *et al.,* 2019 and Chuan He Lab, Science, 2019 with BioRender

The most prevalent mRNA chemical modification is adenosine methylation at the nitrogen-6 position (N6-methyl adenosine or m^{6}A), originally identified in mRNAs in the 1970s. Recently, transcriptome-wide m^{6}A site mapping has shown that this modification is present in thousands of transcripts, and it is uniquely distributed and conserved around stop codons, as well as, at 3'-Untranslated Regions (3'-UTRs) (Dominissini *et al.*, 2012).

1.3 – m^{6}A modifications

m^{6}A modification is a dynamic (reversible) and complex process catalysed by an m^{6}A methyltransferase multicomponent complex that consists of methyltransferase-like protein 3 (METTL3) and methyltransferase-like protein 14 (METTL14) as essential components to facilitate RNA binding (Bokar *et al.,*1997; Liu *et al.,* 2014; Wang *et al.,* 2016; Wang *et al.,* 2016). Depending on the activity and specificity of the m^{6}A methyltransferase multicomponent complex, different subunits can be linked to the stable core of the complex, amongst which are WTAP (Wilms tumour 1 associated protein) and VIRMA (vir like m^{6}A methyltransferase associated), thus creating what is known as the writer complex (Zaccara *et al.*, 2019) (Figure 6).

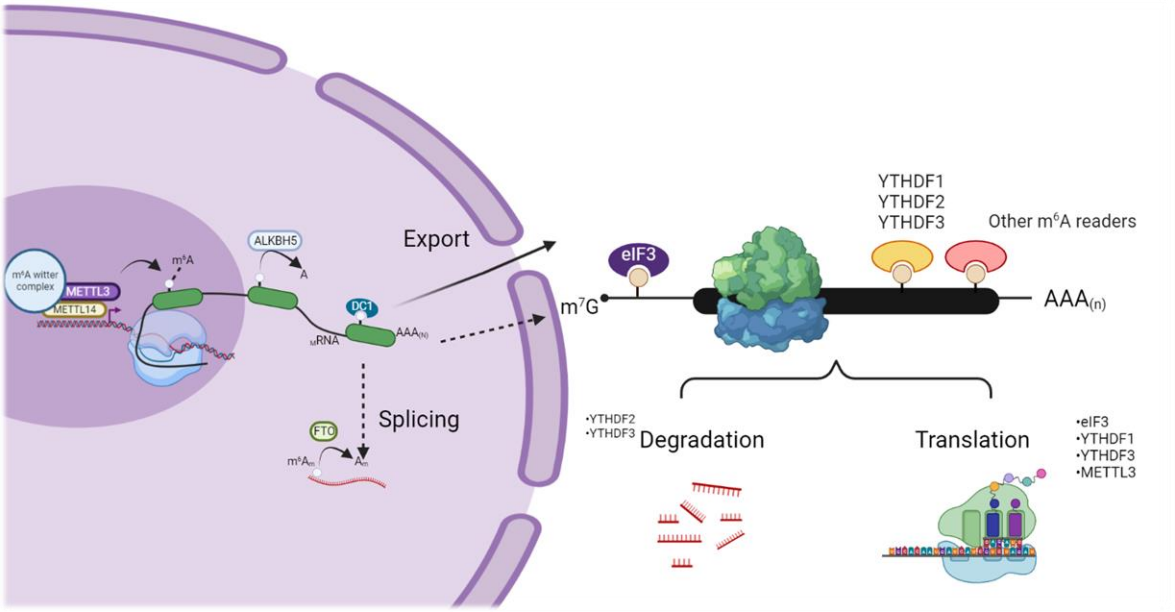


Figure 6 – The m^{6}A mRNA life cycle. RNA-binding proteins (nuclear and cytosolic) recognize modified RNA and mediate downstream effects. While readers in the nucleus have effects on splicing and nuclear export; readers in cytoplasm are linked to mRNA localization, translation efficiency and mRNA stability; however, the exact functions are still under debate. Adapted from: Zaccara *et al.,* 2019 with BioRender.

After being placed, the m^{6}A marks may be removed by RNA demethylases, called erasers, which include the enzymes FTO (FTO alpha-ketoglutarate dependent dioxygenase) and ALKBH5 (alkB homolog 5, RNA demethylase). These erasers take part in distinct biological pathways, interacting with different protein partners. While FTO is responsible for the demethylation of internal m^{6}A and cap-m^{6}A of mRNAs in the cytoplasm and in the cell nucleus (Wei *et al.,* 2018), ALKBH5 mediates demethylation of 3'UTR-m^{6}A, modulating splicing and stability in male germ cells (Tang *et al.,* 2018), being also involved in cancer cell pathways in breast cancer (Zhang *et al.,* 2016) and glioblastomas (Zhang *et al.,* 2017).

The m^{6}A marks are recognized by RNA-binding proteins known as readers that bind m^{6}A-modified mRNA and, in this way, influence their fate regarding stability, translation, splicing and nuclear export (Zaccara *et al.*, 2019). The characterization of this class of effector proteins brought invaluable insights to the understanding of m^{6}A-mediated post-transcriptional gene regulation. Amongst the most direct and robust classes of m^{6}A readers are the proteins containing the YT521-B homology (YTH) domain family, which recognize m^{6}A in a methylation-dependent manner (Hsu *et al.,* 2017; Shi *et al.,* 2017). This cluster of reader proteins includes the YTH domain family of proteins (YTHDF), comprising the highly similar YTHDF1, YTHDF2 and YTHDF3 paralogues, and YTH

domain containing (YTHDC)1–2, with each protein leading to different mechanistic outcomes for the cell.

While YTHDF2, normally present in the cell's cytoplasm, promotes target degradation by recruiting a deadenylase complex (CCR4-NOT) (Wang *et al.,*2014), under the right conditions, such as heat shock, YTHDF2 can lose its cytoplasmatic location, redistributing to the nucleus, becoming upregulated in the nucleus (Zhou *et al.,* 2015). It has also been suggested that nuclear YTHDF2 could compete with FTO to prevent demethylation at 5'UTR-m^{6}A, hence, enhancing cap-independent translation of heat shock response genes in the cytosol (Zhou *et al.,*2015). As for YTHDF1 and YTHDF3, they are involved in promoting target translation, through the recruitment of initiation factors (Wang *et al.,* 2015; Shi *et al.,* 2017). Furthermore, YTHDF3 is believed to be important for the function of both YTHDF1 and YTHDF2, since the knockdown of YTHDF3 results in decreased RNA-binding specificity of the other readers (Shi *et al.,* 2017). According to Shi *et al.,* 2017, YTHDF3 is pivotal at an earlier stage of the RNA life cycle when compared with other YTHDF members. In the cytosol (Figure 6), after being exported from the nucleus, the m^{6}A mark in the mRNA is recognized by YTHDF3–YTHDF1 complex, or by YTHDF3 alone, which facilitates YTHDF1 binding for protein translation enhancement; at this point, YTHDF2 can also bind the modified mRNA for accelerated decay. It is due to this interaction with YTHDF1–2, that YTHDF3 can be further considered as a "buffering agent" for YTHDF1 and YTHDF2 to access their targets (Shi *et al.,* 2017). Regarding the proteins with YT521-B homology, YTHDC1, it is said to play multiple roles in the cell, ranging from the regulation of mRNA splicing to export and acceleration of the decay of some transcripts (Xiao *et al.,* 2016; Shima *et al.,* 2017; Roundtree *et al.,* 2017), whereas YTHDC2 mediates mRNA stability and translation (Hsu *et al.,* 2017).

Opposed to the binding behaviour of YTH domain-containing proteins, other groups of reader proteins can bind m^{6}A-marked RNAs by recognizing RNA binding domains (RBDs), such as K homology (KH) domain, RNA recognition motif (RRM) domains and arginine/glycine-rich (RGG) domains. In some of these cases, the RNA secondary structure can be redesigned by the presence of m^{6}A, thus allowing RNA-protein interactions (Liu *et al.,* 2015). This alternative group of reader proteins includes several heterogeneous nuclear ribonucleoproteins (HNRNPs), which regulate alternative splicing and target transcript processing, as well as, the Fragile X mental retardation protein (FMRP), that impacts RNA translation and stability. Recent studies also suggest

that FMRP could interact with YTHDF1 and YTHDF2 (Edupuganti *et al*., 2017; Zhang *et al.*, 2018).

It is clear that m^{6}A modification affects mRNA stability and translation, as well as RNA splicing and export (Dominissini *et al.*, 2012; He *et al.*, 2021), with an unanticipated impact in cellular phenotypes that is enthusiastically being uncovered.

1.4 – m^{6}A modifications in GC

The number of publications addressing the m^{6}A role in cancer has been increasing rapidly, particularly since 2019, making it clear that epitranscriptome research is an emerging field with promising potential. Of late, m^{6}A modification, or the enzymes involved in its deposition, have started to be associated with a variety of cancers (Lobo *et al.*, 2019), including GC.

In GC, m^{6}A modification has been associated with a more aggressive phenotype *in vitro* (Lin *et al.,* 2019; Yue *et al.,* 2019; Liu *et al.*, 2020) and METTL3 expression in the tumours has been associated with poor patient prognosis as it promotes GC angiogenesis and glycolysis by increasing the stability of *HDGF* mRNA, and activating the AKT signalling pathway (Guan *et al.*, 2020; Wang *et al.,* 2020). FTO and ALKBH1 high expression (at mRNA level) implies a poor prognosis of GC through mining TCGA database (Li *et al.,* 2019). ALKBH5 was shown to promote invasion and metastasis of GC by decreasing methylation of the lncRNA *NEAT1* (Zhang *et al.,* 2019). Some studies demonstrated that, another reader, IGF2BP3, functioned as an oncogene to promote tumour progression in GC (Kim *et al., 2014;* Lee *et al.,* 2017). Knockdown of METTL14 (m^{6}A suppression) promotes GC development by activating the Wnt/PI3K-AKT signalling pathway, whereas increasing m^{6}A levels reversed these molecular and phenotypical changes (Zhang *et al.,* 2019).

When considering all the various outcomes for a cell, that differ with the reader or complex of reader enzymes involved, it becomes clear that the understanding of the expression, function and specificity of the reader enzymes is key to fully uncover the mechanistic basis of m^{6}A effects in GC cells and to reach a more realistic interpretation of the role of m^{6}A modification, and its players, in GC. Furthermore, reader enzymes are a more viable therapeutic target due to their cytoplasmatic location. Thus, by

understanding the non-genetic regulatory mechanisms in cancer cells, future exploitable therapeutic vulnerabilities could be identified and used in improving treatment.

1.5 – Preliminary results

Before the start of the work for this thesis, other elements of the research group initiated an effort to assess the relevance of the reader enzymes in GC. In order to do so, an *in-silico* analysis of the expression of these proteins in GC, followed by a comparison of the obtained results with normal gastric mucosa was performed. Though the expression of all three proteins analysed was higher in GC than in normal gastric mucosa, YTHDF3 had the highest and most statistically significant differential expression (Figure 7A). When evaluating the mRNA expression of YTHDF1–3, in a panel of commonly used GC cell lines, YTHDF3 appeared as the most abundantly expressed reader in all the tested cell lines (Figure 7B).

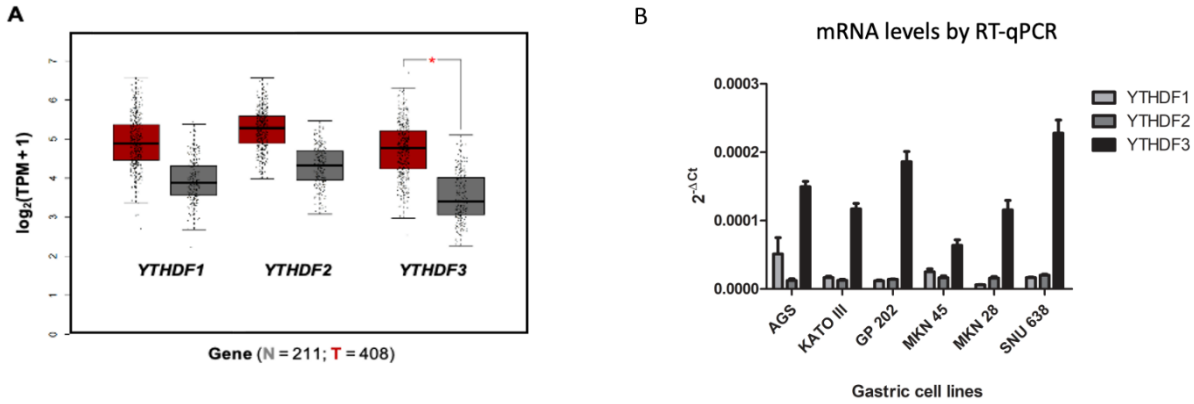


Figure 7 – Expression of YTHDF1–3 "reader" proteins in GC and GC cell lines. (A) Expression of YTHDF1–3 was compared between normal gastric mucosa (N) and GC (T) using data deposited in the TCGA and GTEx databases. Comparison was performed using the free online software GEPIA (http://gepia2.cancer-pku.cn). Results were considered significant when *P-value < 0.01. (B) Expression of YTHDF1–3 was determined, in a panel of GC cell lines, using qRT-PCR.

Next, to determine the relevance of YTHDF3 in GC we generated two knock-out (KO) cell lines for YTHDF3 by CRISPR/Cas9. For KO production, AGS and SNU638 cell lines were transfected with the chosen sgRNA, in order to delete the fragment containing the

YTH-domain. After transfection, the cells underwent single-cell sorting to generate monoclonal cultures of ΔYTHDF3 cells.

Survival of single-cells in culture diverged between cell lines, with AGS being more sensitive to transfection than SNU638. Due to the low growth efficiency, only one clone of each cell line was selected. Once selected and sequenced, the ΔYTHDF3 cell lines were assessed by Western-blot (Figure 8), where no YTHDF3 protein was detected.

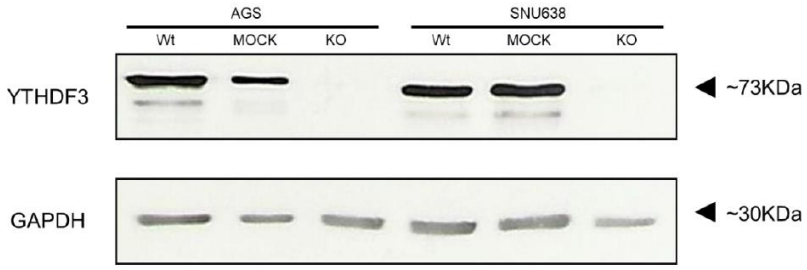


Figure 8 – Western-blot analysis of CRISPR YTHDF3 knock-out in AGS and SNU638 cell lines. GAPDH protein was used as housekeeping gene.

With the successful knock out of YTHDF3, a change in the morphology of both ΔYTHDF3 cell lines was clearly observed. AGSΔYTHDF3 exhibited a decrease in abundance and size of AGS-characteristic giant cells, while SNU638ΔYTHDF3 showed a decrease in the number of filopodia and lamellipodia protrusions, as well as a rounder appearance (Figure 9A).

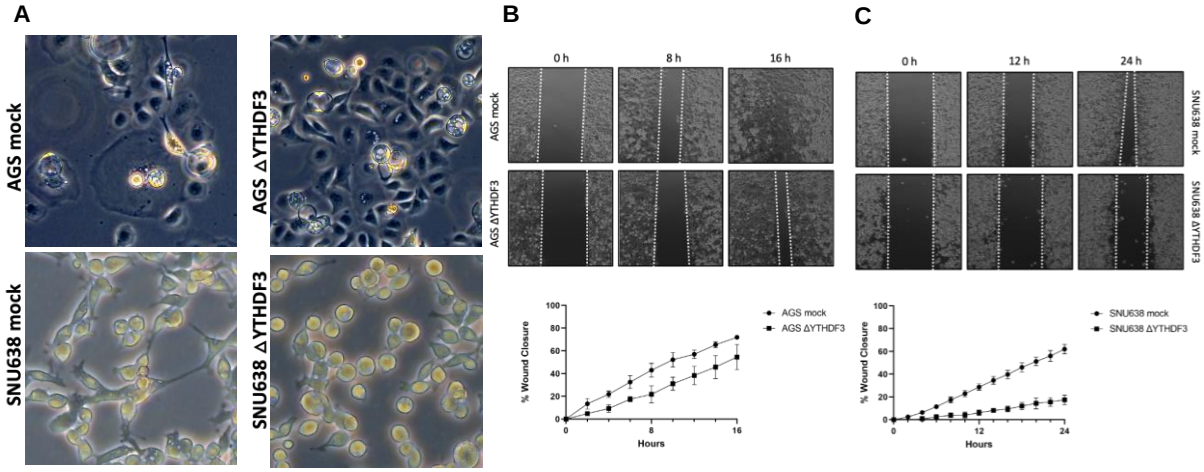


Figure 9 – Phenotypic alterations after YTHDF3 deletion in GC cell lines. (A) Morphology of AGS mock, AGSΔYTHDF3, SNU 638 mock and SNU638ΔYTHDF3 cells. Megalocytic cells in AGS mock and filopodia and lamellipodia protrusions in SNU mock cells are indicated with arrows. (B) and (C), Wound Healing Assay. Representative images at different time points

Since the altered morphological features of the ΔYTHDF3 cells could also be associated with cell motility, a wound healing assay was performed to evaluate the ability of the cells to move and migrate. The results obtained showed a clear decreased ability of the ΔYTHDF3 cells to repair the wound, compared to wild-type cell lines, particularly evident for SNU638 (Figure 9B and Figure 9C).

2 – Objectives

Having demonstrated that YTHDF3 plays a role in the biological behaviour of GC cells and despite it being the reader with highest expression, we cannot rule out that the other readers might also have some function. Thus, as a result of the lack of evidence of the specialized effects of the YTHDF proteins on the m^{6}A-modified mRNA in GC, this project focused in understanding the level of functional overlap between these three proteins using two GC cell lines as models.

In order to shed some light on the major aim, three separate objectives were drawn:

Objective 1 - To characterize the expression of YTHDF1/2/3 in two wild-type cell lines and in two cell lines that lack YTHDF3 (previously generated by CRISPR/Cas9), using qPCR.

Objective 2 - To downregulate YTHDF1 and 2 expressions in wild-type cell lines, using siRNAs, and evaluate the resultant phenotype.

Objective 3 - To study the expression of YTHDF1 and 2 in TMAs, of normal tissues, and compare it with the previously obtained pattern for YTHDF3.

3 – Methods

3.1 – Cell culture

Human GC cell lines AGS and SNU638 were used in this work (either on their wild-type form, mutant, with deletion of YTHDF3, or the respective mock form). They were thawed in RPMI-1640+GlutaMAX™ (Thermo Fisher Scientific, MA, USA) supplemented with 20% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere. GC cells were seeded in T-75 flasks and maintained in the medium mentioned above supplemented with 10% FBS until a confluence of 60–70% was reached. At that point they were trypsinized with 0.05% Trypsin-EDTA (1X) (Thermo Fisher Scientific, MA, USA) and sub-cultured. For all the experiments, cells with nine or higher number of sub-cultures where discarded and more recent vials were thawed.

3.2 – mRNA extraction and real-time PCR (polymerase chain reaction)

YTHDF1, YTHDF2 and YTHDF3 mRNA expression was analysed by real-time PCR. RNA was firstly extracted from the four GC cell lines (AGS, SNU638, AGSΔYTHDF3, SNU638ΔYTHDF3) using TRI Reagent® (Sigma-Aldrich, MO, USA), according to manufacturer's instructions. RNA concentration was determined using NanoDrop™1000 (Thermo Scientific, CA, USA) and purity was determined using the 260/280nm and 260/230nm absorbance ratios.

cDNA was obtained from 2 µg of RNA, by a reverse-transcription reaction using the SuperScriptIV™ Reverse Transcriptase (Thermo Scientific, CA, USA) and following the manufacturer's instructions.

100ng of cDNA product was used for the real-time PCR reaction, together with the *Power* SYBR™ Green PCR Master Mix (Thermo Scientific, CA, USA) and the primers listed on Table 2, on a 7500 Fast Real-Time PCR System (Thermo Scientific, CA, USA).

Table 2 – Primers used for real-time PCR

Primer ID	Primer Sequence (5' to 3')
YTHDF1_Forward	5'-CCTACAAGCACACAACCTCCA-3'
YTHDF1_Reverse	5'-CGCAAGGAACGGCAGAGTC-3'
YTHDF2_Forward	5'-CCAGCTACAAGCACACCACT-3'
YTHDF2_Reverse	5'-AAAGGAACGTCAAGGTCGTGG-3'
YTHDF3_Forward	5'-TGCTACTTTCAAGCATACCACCT-3'
YTHDF3_Reverse	5'-GCCATGCGTAGGGAGAGAAA-3'
18S_Forward	5'-CGCCGCTAGAGGTGAAATTC-3'
18S_Reverse	5'-CATTCTTGGCAAATGCTTTCG-3'

The real-time PCR conditions are shown in Table 3 and followed the manufacturer's recommendations.

Table 3 – Real-time PCR parameters

Step	Temperature	Duration	Cycles
Enzyme activation	95 °C	2 minutes	
Initial Denaturation	95 °C	10 minutes	
Denaturation	95 °C	15 seconds	45x
Annealing/extension	60 °C	1 minute	

A dissociation step, for melting curve analysis, was obtained at the end of the amplification by increasing the temperature from 60°C to 95°C with a ramping rate of 100% for 15 seconds and decreasing again the temperature to 60°C for 1 minute with a ramping rate of 100%. The dissociation was read after this stage by increasing once again the temperature to 95°C with a ramping rate of 1% for 30 seconds. To end the dissociation step, the temperature decreased again to 60°C with a ramping rate of 100% for 15 seconds.

Data analysis was performed using *18S* rRNA to normalize the expression and following the comparative ΔΔCt method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

3.3 – mRNA-sequencing

Three paired replicates of SNU638 mock and SNU638ΔYTHDF3 cells were used for RNA extraction and sequencing. Total RNA extraction using PureLink™ RNA Mini Kit (Thermo Fisher Scientific) was performed according to manufacturer's instructions. Quantification and quality control of total RNA were assessed using the NanoDrop ND-1000 (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent Technologies) systems, and only samples with an RNA integrity number above eight were considered for further study. Preparation of RNA library and transcriptome sequencing was outsourced to Novogene (UK). Genes with adjusted p-value < 0.05 and | log2(FoldChange) | > 1 were considered as differentially expressed.

3.4 – Cell Viability

Cell Viability was measured by PrestoBlue™ Cell Viability (PB) assay (Thermo Scientific, CA, USA) according to manufacturer's instructions. Here, 10^5 cells/well were seeded into a 24-well plate in complete media and incubated for 24h. After 24h, media was changed to RPMI 5% FBS, and cells were incubated for another 48h before adding PB. Cells were incubated in the presence of PB at 37ºC for 45 minutes and the absorbance values at 560nm (and 600nm as reference) were obtained using a Synergy™ 2 Microplate reader from BioTek. The assay comprised 3 independent experiments for each cell line, with 3 technical replicates in each experiment. All statistical analysis was performed using Microsoft Excel and GraphPad Prism version 8.0.1 for Windows.

3.5 – Protein extraction and Western blotting

Cell pellets were rinsed with PBS at 4°C, resuspended in RIPA buffer (150mM NaCl, 50mM Tris, 1% NP-40, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Switzerland), 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate (Na_3VO_4), 20nM sodium fluoride (NaF), and incubated on ice for 30 minutes. The samples were then centrifuged at 16000 g for 15 minutes at 4ºC. Supernatants were collected for protein quantification by the BCA method (Pierce BCA Protein Assay Kit, Thermo Scientific). Volumes were adjusted with RIPA buffer in order to obtain the same

protein concentration in all samples (10 µg). The absorbance readings at 562 nm were performed using a Synergy™ 2 Microplate reader from BioTek.

For Western-blot analysis, samples were denatured in Laemmli buffer at 95°C for 5 minutes, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the appropriate antibodies after they were blocked with either 5% non-fat milk or 5% bovine serum albumin (BSA) (Sigma-Aldrich) in TBS-1% Tween-20 (Sigma-Aldrich) for 1h at RT. Incubation with primary antibodies (Table 4) proceeded overnight at 4°C.

Table 4 – Primary antibodies

Antibody	Dilution	Code	Manufacturer
YTHDF1	1:2500	ab252346	abcam, Cambridge, United Kingdom
YTHDF2	1:2000	ab220163	
YTHDF3	1:2000	ab103328	
MISP	1:2000	HPA062232	Sigma-Aldrich, Missouri, United Sates of America
Actin	1:100	SC47778	Santa Cruz Biotechnology, Texas, United Sates of America

Afterwards, membranes were washed with TBS-T (3x10 minutes each) and then incubated with a suitable horseradish peroxidase-conjugated secondary antibody for 1h at RT. Membranes were resolved using enhanced chemiluminescence substrate (ECL) and bands were detected and quantified in a ChemiDoc imaging system using ImageLab 6.0.1.

3.6 – Immunohistochemistry (IHC)

Tissue microarrays (TMAs) were created using formalin-fixed cores, previously obtained from paraffin-embedded normal tissue blocks from the surgical specimens. TMAs were sectioned in a microtome (Microm HM335E) with a 4µm thickness, and the expression of YTHDF1, YTHDF2 and YTHDF3 was evaluated by IHC staining, using the protocol described in Mesquita *et al*.,2019's where tissues were deparaffinised and hydrated. Heat-induced epitope retrieval was carried out in an IHC-Tek^{TM} Epitope Retrieval Steamer Set, for 40 minutes in 10mM Tris-EDTA pH9.0. Activity of endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes. Primary antibodies

(anti-YTHDF1 1:100 (ab252346); anti-YTHDF2 1:600 (ab220163); anti-YTHDF3 1:100 (ab103328), abcam, Cambridge, United Kingdom) were incubated at 4°C overnight. Detection was done using Dako REAL Envision Detection System Peroxidase/DAB+ (Dako, Glostrup, Denmark). Then, sections were counterstained with hematoxylin, dehydrated and mounted. Samples were classified from Low to High based on the percentage of stained cells (High > 20%), upon agreement of 3 observers.

4 – Results

Following the hypothesis that m^{6}A mRNA modifications, particularly their readers, play a role in GC progression and heterogeneity, we tried to understand the level of functional overlap between the three proteins.

4.1 – Characterization of YTHDF1/2/3 expression in normal tissues

We started by characterizing the expression of YTHDF1/2/3 in a variety of normal tissues, by means of TMAs. We observed that the expression of these proteins is mostly cytoplasmatic (Appendix I). Nevertheless, in some tissues some nuclear expression could also be detected (Figure 10). The reader YTHDF1 was the one with a more restricted expression profile being absent from liver, heart, cerebellum, lymph node and spleen. YTHDF2 and 3 had a more similar expression profile, but nevertheless YTHDF2 expression could not be detected in the liver and in heart (Figure 10). All three proteins were expressed in oesophagus, small intestine, breast, thymus, gallbladder, and tongue (Figure 10 and Appendix I)

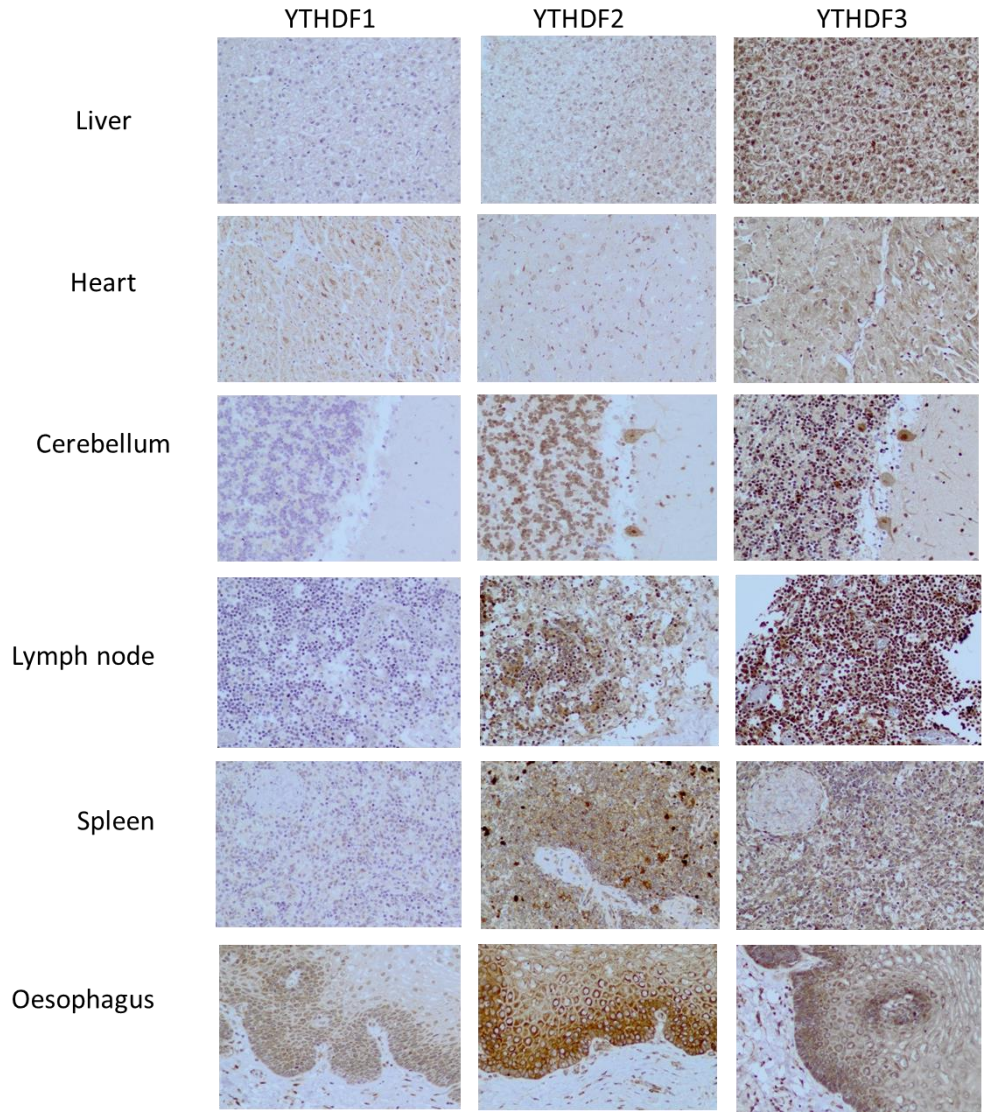


Figure 10 – Expression of the enzyme YTHDF1–3 was evaluated in normal tissue with TMA by immunohistochemistry (YTHDF1 - ab252346; YTHDF2 - ab220163; YTHDF3 - ab103328). Magnification x200.

4.2 – Downregulation of YTHDF1/2/3 expression and evaluation of the resultant phenotype

Next, we focused our study in GC cell lines. In order to understand the level of redundancy of the reader proteins YTHDF1/2/3 we started by characterizing their expression in the GC cell lines AGS and SNU638, to get baseline information on the expression profile of these proteins in each cell line (Figure 11). The results showed that SNU638 has a higher expression level than AGS of YTHDF2 and 3, whereas AGS presents a slightly higher expression of YTHDF1.

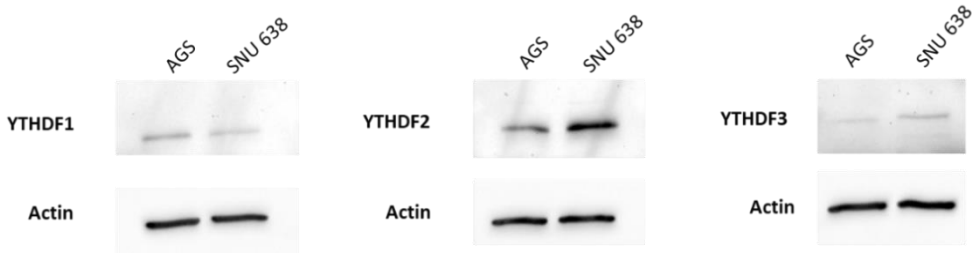


Figure 11 – Protein expression detection of YTHDF1, YTHDF2 and YTHDF3 by Western-blot in the AGS and SNU638 wild-type lines.

Next, we downregulated their respective expression using siRNAs and determined the impact in cell viability (Figure 12A, Figure 13A). In AGS cells, the downregulation of YTHDF1 and 3 was efficient but downregulation of YTHDF2 was not so efficient with significant variation between experiments. Yet, the viability results indicate that AGS cell line is sensitive to the downregulation of any of the three readers exhibiting a significant reduction in cell viability of ~22% upon downregulation of YTHDF1 and ~33% upon downregulation of YTHDF2 and YTHDF3. (Figure 12B).

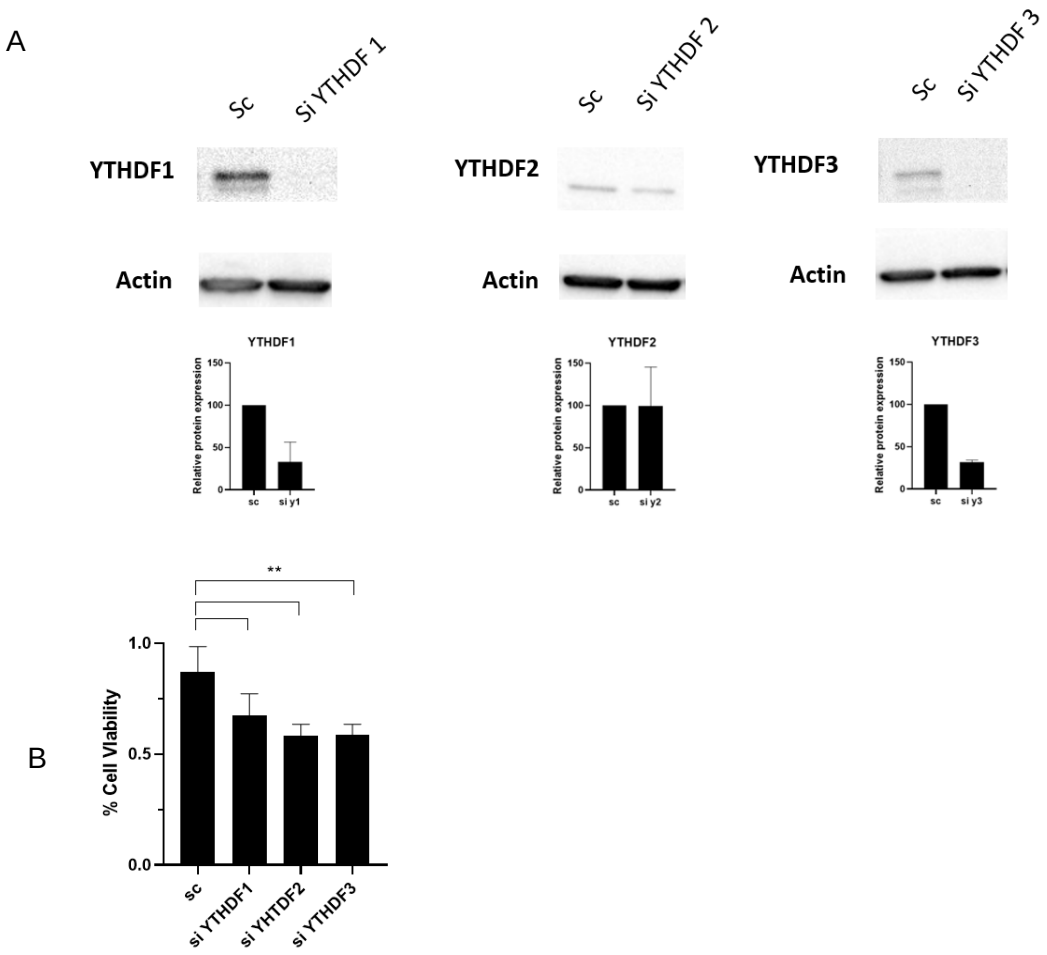


Figure 12 – Expression of YTHDF1–3 "reader" proteins in AGS cell line after downregulation (A) and effect of YTH readers knockdown in AGS viability (B). The results were normalized to the scRNA condition. (n=3, P<0.01. Expression of YTHDF1–*3* in B was determined by Western blotting using a rabbit anti-YTHDF1–3 antibodies respectively, β-Actin was used as loading control. The graphical illustration of the protein quantification was made to clarify the results shown of the gel.

For SNU638 cell line the only transfection that worked was that for YTHDF3, as this cell line proved to be much more difficult to transfect with siRNAs which resulted in a less efficient downregulation of each of the three readers. As a consequence, in the viability assay, though a decrease in viability is visible, particularly with YTHDF3 downregulation, this decrease is not significant (Figure 13B).

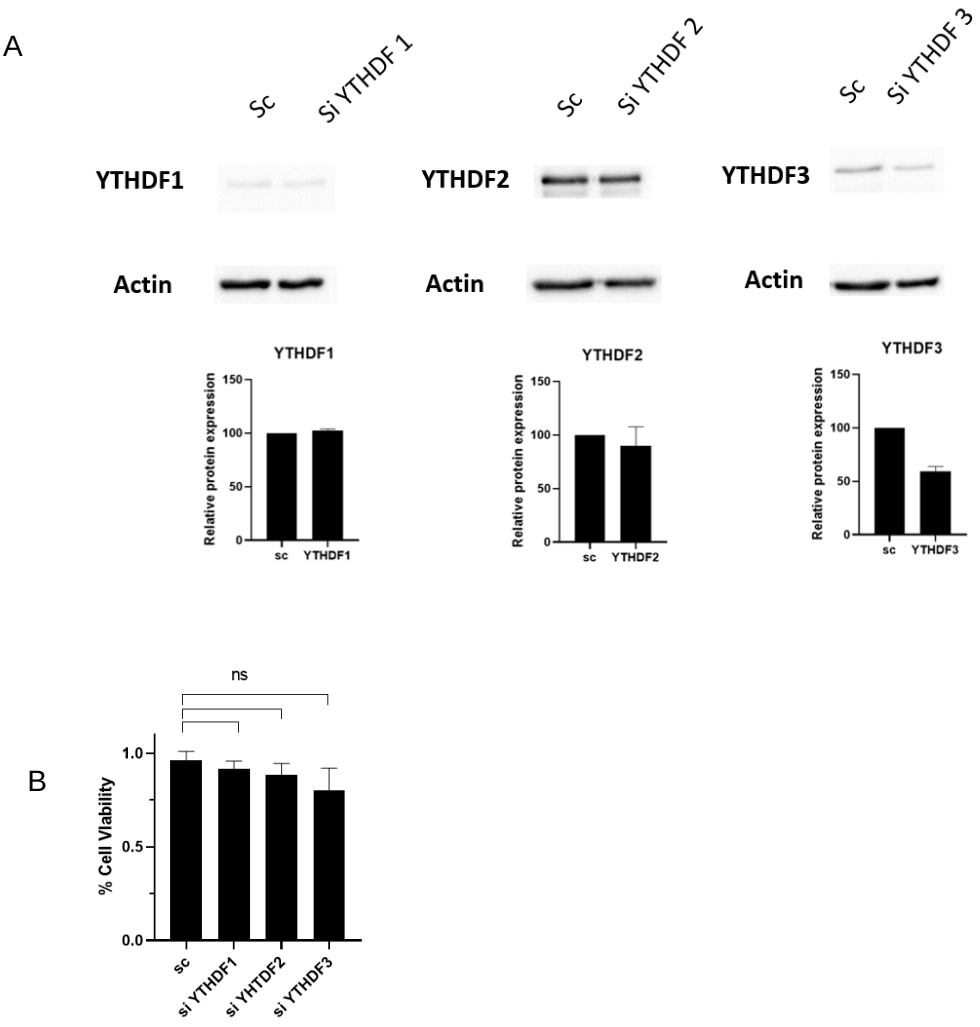


Figure 13 – Effect of YTH readers knockdown in SNU638 viability (A), expression of YTHDF1–3 "reader" proteins in SNU638 cell line, after downregulation (B). The results were normalized to the scRNA condition (n=3; ns=non-significative result). Expression of YTHDF1–3 in B was determined by Western blotting using a rabbit anti-YTHDF1–3 antibodies respectively, β-Actin was used as loading control. The graphical illustration of the protein quantification was made to clarify the results shown of the gel.

4.3 – Characterisation of YTHDF1/2/3 expression in YTHDF3KO cell lines

Since the three YTHDF proteins are very homologous, and exhibited some degree of redundancy in expression and also in cell viability in AGS cell lines, we hypothesised that a compensation phenomenon could take place in the YTHDF3 knock-out cells. So we assessed whether YTHDF3 KO had an impact in the expression levels of the other two reader proteins, using quantitative Real-time PCR (Figure 14) and Western-blot. At the mRNA level, it was observed that within cell lines there were no alterations in the expression of the YTHDF1 or YTHDF2 (Figure 14).

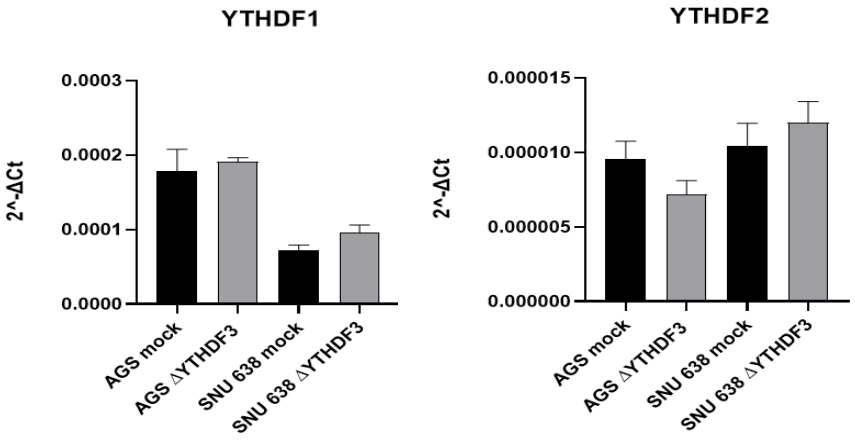


Figure 14 – Expression levels of YTHDF1–2 for each cell line, determined by real time PCR. Within cell lines there were no significant alterations on the expression of the YTHDF1 or YTHDF2.

In order to validate the results obtained with the real-time PCR, we also confirmed the effect at the protein expression level by Western-blot analysis (Figure 15). The results showed that both cell lines, mock and ΔYTHDF3 versions, express the two reader proteins. Focusing on YTHDF1 protein (Figure 15A), AGSΔYTHDF3 shows an increased protein expression when compared with the mock counterpart, while in SNU638 no alteration in protein level was detected. The same trend was observed for YTHDF2 (Figure 15B).

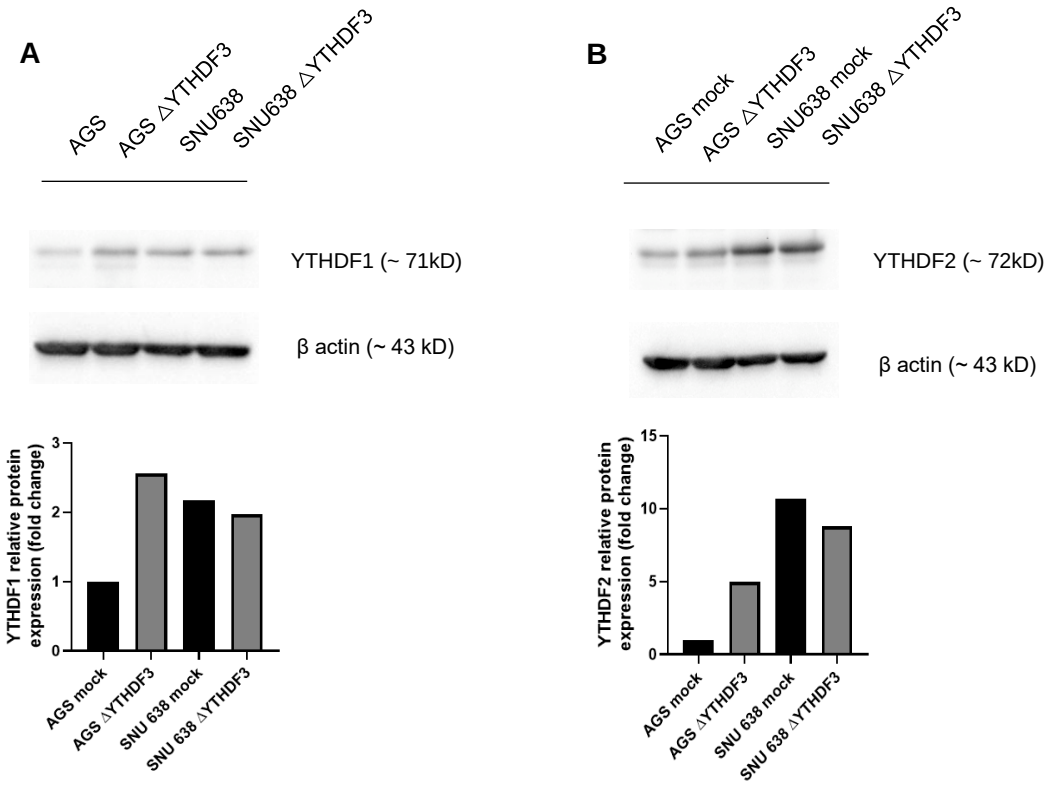


Figure 15 – Protein expression detection of YTHDF1 (A), YTHDF2 (B) by Western-blot in the AGS and SNU638 mock and ΔYTHDF3, after the YTHDF3 knock-out.

Overall, the results show that YTHDF1/2 expression is present in GC and at the protein level there is an increase in the expression of YTHDF1 and 2 in ΔYTHDF3 mutants, however the same is not observed at the mRNA level suggesting that there is no compensatory mechanism regarding their expression.

4.4 – mRNA-sequencing, downregulation of YTHDF1/2 expression and evaluation of the MISP values

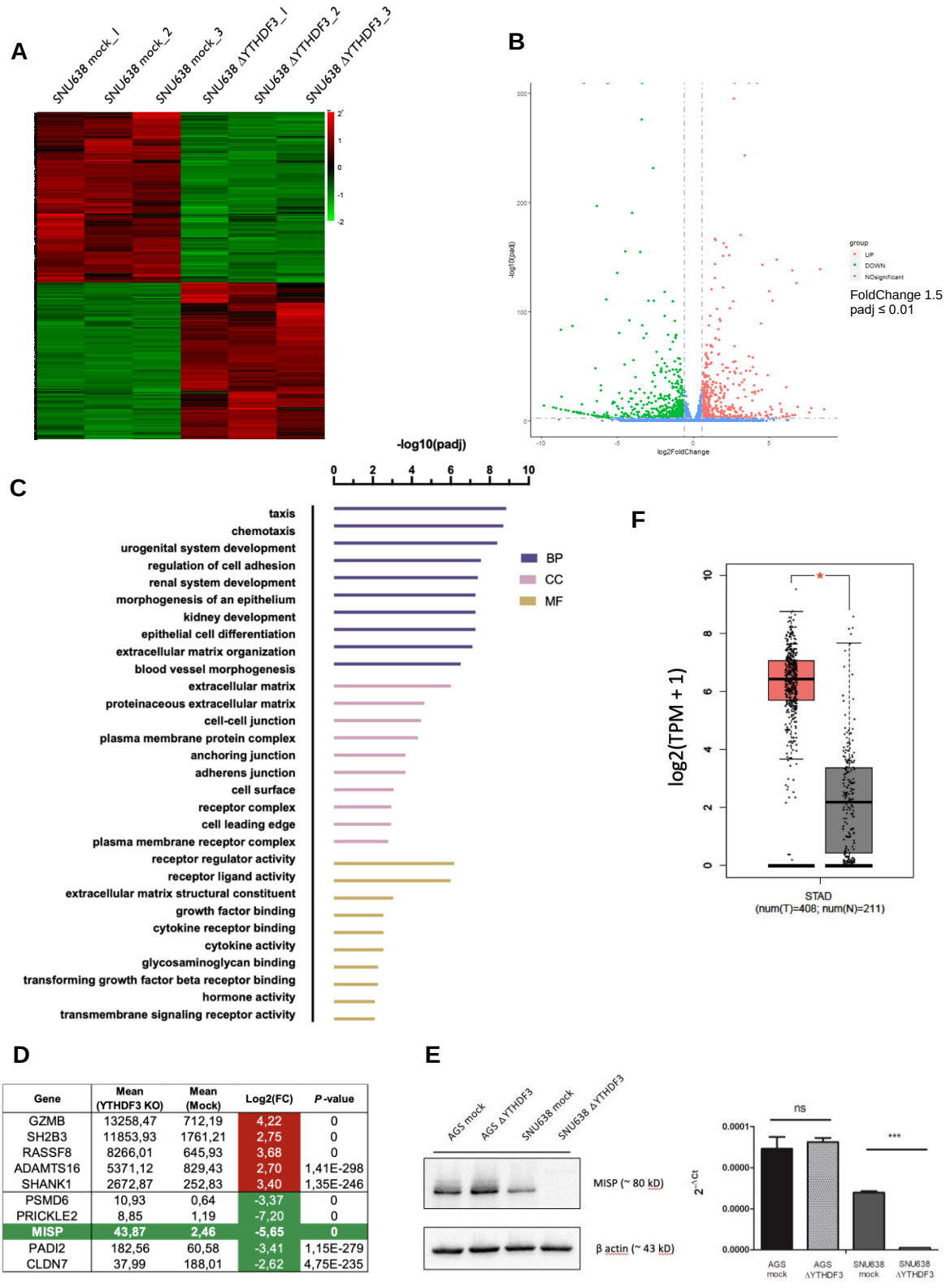
In order to identify the molecular targets of YTHDF3, which could be the molecular link between YTHDF3 and the alterations in cell morphology and motility in GC, mRNA-sequencing was performed in SNU638ΔYTHDF3, and in SNU638mock (Figure 16).

Knocking out YTHDF3 was shown to have significant changes in the transcriptome (Figure 16A), with a total of 589 genes being downregulated and 546 becoming upregulated in SNU638ΔYTHDF3 (Figure 16B).

GO analysis revealed several cellular mechanisms affected by YTHDF3 (Figure 16C), including pathways related with cellular-extracellular matrix interactions, epithelial morphology, and motility. Of the genes most significantly altered was the Mitotic Spindle Positioning (MISP) (Figure 16D), that appeared as one of the most significantly downregulated in SNU638ΔYTHDF3. We further demonstrated that MISP expression was lost in SNU638ΔYTHDF3, but not in AGSΔYTHDF3 (Figure 16E).

With MISP being associated with mitotic spindle orientation and centrosome clustering, as well as with inducing stress fibres and other thick actin filaments (Kumeta *et al.,* 2014), which are directly correlated with cell migration (Maier *et al.,* 2013), MISP was considered a promising target to explain the phenotype observed in ΔYTHDF3 cells.

At this point, an analysis of the TCGA database showed that MISP is overexpressed in GC (Figure 16F), with the most recent literature connecting it to poor prognosis in different neoplasias (Huang *et al.,* 2022).



Gene	Mean (YTHDF3 KO)	Mean (Mock)	Log2(FC)	*P*-value
GZMB	13258,47	712,19	4,22	0
SH2B3	11853,93	1761,21	2,75	0
RASSF8	8266,01	645,93	3,68	0
ADAMTS16	5371,12	829,43	2,70	1,41E-298
SHANK1	2672,87	252,83	3,40	1,35E-246
PSMD6	10,93	0,64	-3,37	0
PRICKLE2	8,85	1,19	-7,20	0
MISP	**43,87**	**2,46**	**-5,65**	**0**
PADI2	182,56	60,58	-3,41	1,15E-279
CLDN7	37,99	188,01	-2,62	4,75E-235

Figure 16 – Loss of YTHDF3 leads to MISP downregulation and mislocalization. (A–F) RNA-seq analysis was performed in SNU638ΔYTHDF3 (n=3) and their mock counterparts (n = 3). (A) K-means clustering was performed on differentially expressed genes (DEGs) and is presented as a heatmap (upregulated and downregulated). (B) Volcano plot showing fold-change and P-value distribution for the list of detected transcripts in the SNU638ΔYTHDF3 versus mock cells. (C) GO Analysis of DEGs concerning the associated biological processes. The 10 most significant terms in BP (Biological Processes), CC (Cellular Component), and MF (Molecular Function) are depicted according to their P value. (D) Top DEGs in SNU638ΔYTHDF3. (E) qRT-PCR analysis and proteins expression of MISP in AGSmock, AGSΔYTHDF3, SNU638mock and SNU638ΔYTHDF3 cells. (*** p < 0.001) (F) MISP expression in GC (T) and normal gastric mucosa (N), using data deposited in the TCGA and GTEx databases.

Given potential redundant role of YTHDF1/2/3, we assessed weather downregulating each one of these proteins had the same impact in MISP expression. Thus, we characterized the expression of MISP protein, once YTHDF1/2/3 had been downregulated in AGS and SNU638 cell lines, after cell transfection with the correspondent siRNA. When evaluating the expression of MISP, by Western-blot, the results suggest that downregulation of any of the three proteins in AGS (Figure 17), leads to a decrease in MISP expression, with YTHDF1 showing a decrease of more than 50%, and YTHDF3 being completely absent.

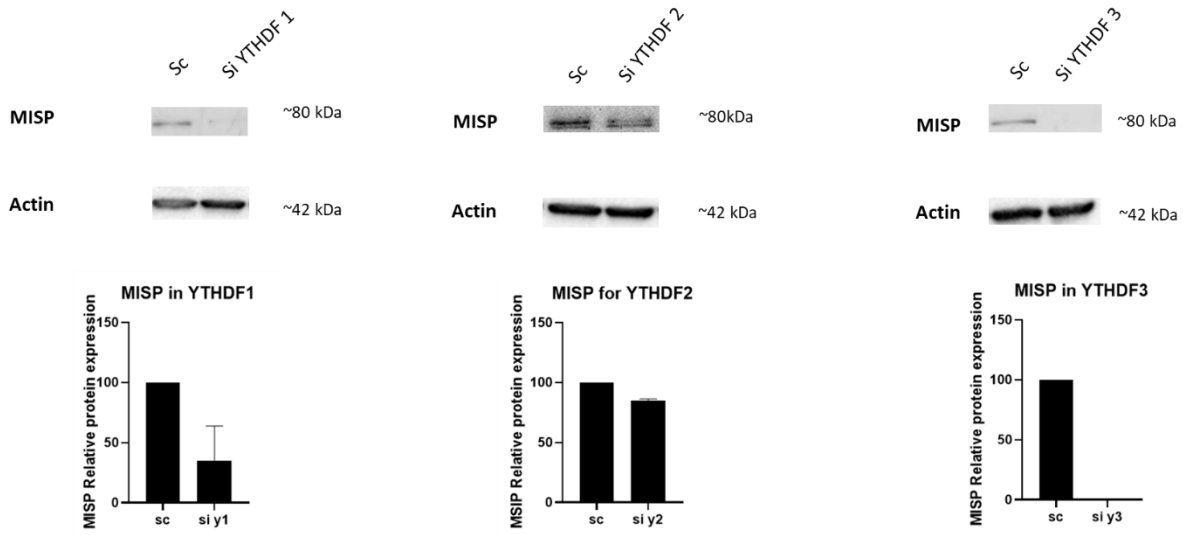


Figure 17 – Expression of MISP protein in AGS cell line, after YTHDF downregulation. Expression of MISP was determined by Western blotting using a rabbit anti-MISP antibody and β-Actin was used as loading control. The graphical illustration of the protein quantification was made so to clarify the results shown in the gel. After downregulation of the YTHDF proteins, MISP suffers a reduction in its expression.

On SNU638, however, MISP expression does not appear to change with the downregulation of the YTHDF proteins (Figure 18).

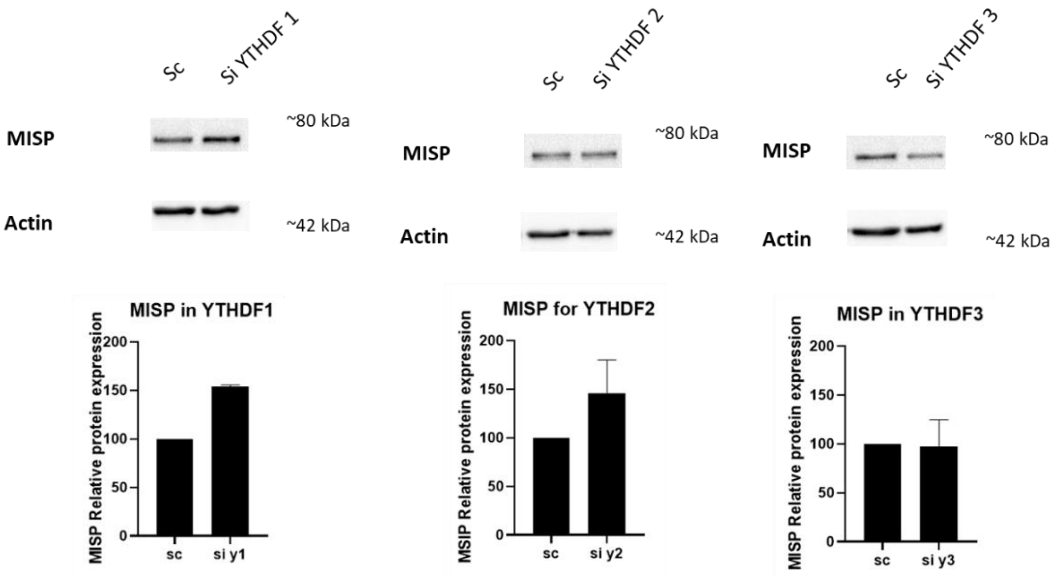


Figure 18 – Expression of MISP protein in SNU638 cell line, after YTHDF downregulation. Expression MISP was determined by Western blotting using a rabbit anti-MISP antibody and β-Actin was used as loading control. The graphical illustration of the protein quantification was made to clarify the results shown of the gel. After downregulating YTHDF1 and YTHDF2, the expression of MISP displays an increase in its expression. While in the case of YTHDF3 downregulation, no alteration on MISP expression is shown.

5 – Discussion

GC remains, to this date, one of the leading causes of cancer mortality worldwide, with an alarming 5-year survival rate of less than 35% (Sung *et al.,* 2021). This dismal prognosis is in part due to a small number of therapeutic options as, thus far, the therapeutic approach is determined by the stage of the disease at the time of diagnosis.

Over the last few years, new evidence has confirmed the impact of m^{6}A on fine-tuning and managing gene expression (Zaccara *et al.,* 2019; Destefanis *et al.,* 2021). It has also been shown that m^{6}A participates in some biological processes during malignant tumour development (Lin *et al.,* 2019; Liu *et al.,* 2020). In the beginning, the focus of studies about m^{6}A modifications and human cancer laid in the addition and removal of m^{6}A by writers and erasers, respectively (Chen *et al.,* 2019). Recently, readers have become the focal point of a variety of studies that emphasize epigenetic m^{6}A modifications, especially since only the YTH domain of the YTHDF proteins is able to function in the binding process to the m^{6}A modification, which suggests that the YTH domain might be responsible for the recognition of mRNA targets (Shi *et al.,* 2021). However, there are a series of questions regarding YTHDF readers that still need answering - from the overlapping degree of the different readers' targets, to poorly understood selective mechanism of each reader - which might involve preferred motifs, phase separation or the function of YTHDF3 (Shi *et al.,* 2021).

With this in mind, the results presented in this work highlight the relevance of the function of the YTH family of proteins, particularly YTHDF3 as a possible key factor in understanding what m^{6}A modifications may entail in GC.

First, we established as a baseline for future comparison that both in AGS and in SNU638 the three YTHDF proteins are present, with AGS having a higher expression of YTHDF1, while SNU638 shows a greater expression in YTHDF2/3.

To date, it is a known fact that YTHDF family consists of three paralogues with a high level of similarity, due to a high amino acid correspondence over their entire sequence (Patil *et al.,* 2017). Previous studies report contradictory evidence on the specialized effects that the three proteins could have on m^{6}A marked mRNAs. While some studies report on a specific effect on m^{6}A by each protein (YTHDF1 – enhances translation of m^{6}A modified mRNAs; YTHDF2 – promotes degradation; YTHDF3 – combines both functions) (Whang *et al.,* 2014; Whang *et al.,* 2015; Shi *et al.,* 2017), others state that

YTHDF1/2/3 have similar roles in mRNA degradation either by using reporter RNA degradation (Kennedy *et al.,* 2016) or by mRNA deadenylation assays (Du *et al.,* 2016), stressing that the level of sequence homology between the YTHDF proteins makes it unlikely that each protein has its own function (Zaccara *et al.,* 2019).

When downregulating both cell lines for the YTHDF1/2/3, the effectiveness varied between AGS and SNU638. YTHDF1/3 were successfully downregulated in AGS cells, but not YTHDF2. These findings were supported by the viability assay performed, which indicated AGS as a sensitive cell line to the downregulation of any of the YTHDF proteins with a significant reduction in cell viability. SNU638 displayed less efficient downregulation, as a certain level of technical difficulty comes into play for this particular cell line. SNU638 is a cell line that tends to create cell clusters, making it difficult to count and subculture a similar number of cells on each experiment, which in our case translated in difficulties in transfecting SNU638 efficiently. Nevertheless, the viability assay showed a decrease in cell viability, particularly in YTHDF3 downregulation, though none of the effects were significant. Our results suggested some degree of redundancy in the function of the three YTHDF proteins, which agrees with previous studies (Lasman *et al.,* 2020), and it is the logical outcome when considering these proteins' homology (Wang *et al.,* 2014, 2015; Shi *et al.,* 2017), cellular location and shared targets (Patil *et al.,* 2016, 2018; Li *et al.,* 2017).

Taking into account the homology between YTHDF1/2/3, we decided to evaluate their expression in a previous model that we obtained, where we knocked-out YTHDF3. Once the KOΔYTHDF3 was achieved, an evaluation of a possible compensatory behaviour of proteins YTHDF1/2 was conducted. Here, comparing between mock and ΔYTHDF3 in AGS and SNU638, YTHDF1 and YTHDF2 maintained their mRNA levels within each cell line, while an increase in protein expression levels occurs when comparing AGS mock to AGSΔYTHDF3 (though SNU638 does not exhibit the same trend). Since this experiment was performed only once and the differences were not confirmed on the RNA level, we speculate that it might not reflect a true result. Although it requires further validation, we concluded that, despite belonging to the same YTH domain family, YTHDF proteins do not compensate for the absence of each other. Which could be a possible advantage if YTHDF3 turns out to be a viable therapeutic target. In agreement, previous studies have suggested that, in certain systems, YTHDF readers cannot compensate for each other (Lasman *et al.,* 2020). Thus far, it had been demonstrated how KOΔYTHDF2 alone is sufficient to stop proper oocyte maturation (Ivanova *et al.,* 2017), and how a single KOΔYTHDF1 or ΔYTHDF2 can be the cause of neural defects (Li *et al.,* 2018; Shi

et al., 2018). In any case, it could be argued that this result could be caused by discrepancies in expression levels in the different tissues, which has not been fully addressed thus far.

Apart from allowing to check for protein compensation, the KOΔYTHDF3 also shows alterations of some key mechanisms that are connected to the regulation of the subcellular localization of the actin binding protein MISP.

Thus far, it is well established that the positioning of a cell's division plane is of extreme importance for the morphogenesis and the architecture of the tissue it forms (Théry, & Bornens, 2006). Hence, mitotic spindle orientation ought to be frequently controlled in live tissues. From the various elements interacting in the network responsible for a proper spindle orientation and mitotic progression, MISP has been reported to be the missing link that is involved in correcting mitotic spindle positioning and centrosome clustering, as MISP has been reported to bind to the actin cytoskeleton and co-localize with focal adhesions (Maier *et al.,* 2013). Moreover, it has been demonstrated that MISP subcellular localization (cytoplasmatic versus cortical) must be tightly regulated to ensure proper spindle assembly (Zhu *et al.,* 2013). Interestingly, apart from being implicated in mitotic spindle orientation and centrosome clustering, MISP is involved in inducing stress fibres and other thick actin filaments (Kumeta *et al.,* 2014), which correlate with directed cell migration (Maier *et al.,* 2013) and could be a possible pathway to explain the phenotype observed in ΔYTHDF3 cells. Furthermore, analysis of the TCGA database showed that MISP was overexpressed in GC, and recent literature has linked it to poor prognosis in different neoplasias (Huang *et al.,* 2022). Hence, it can be assumed that YTHDF3, indirectly, regulates cell morphology and migration in GC cell lines, through impairment of the mitotic spindle orientation and the actin cytoskeleton. Additionally, mechanistically, YTHDF3 is also responsible for Ezrin expression levels, which in turn regulates the subcellular localization of the actin binding protein MISP (Kschonsak & Hoffmann, 2018).

Here, we also assessed whether YTHDF1/2/3 had redundant functions in regulating MISP expression. In AGS, the downregulation of all YTHDF proteins leads to different levels of MISP expression reduction, which suggests that YTHDF proteins do have redundant functions in regulating MISP. The disparity between MISP expression on AGS wild-type and AGSΔYTHDF3 might be due to the clonal selection used to create the AGSΔYTHDF3 line. It was observed that AGS cells were very sensitive to YTHDF3 removal, and it could be speculated that the cells that survived have some mechanism that allowed them to compensate for the lack of YTHDF3. We can even relate this with

the increased levels of YTHDF1 and 2 but, as mentioned before, this requires confirmation with replicates of the Western-blot. In SNU638, however, MISP expression does not appear to suffer with the downregulation of the YTHDF proteins. These results may either be the outcome of cell line heterogeneity, but most likely are the result of the technical difficulties that appeared when working with SNU638. In other to confirm the AGS and the SNU638 difference in results, a new assay should be performed with the standard three biological and technical replicas.

These observations, combined with alterations in cell phenotype, and expression levels of each YTHDF protein, reveal an interesting new molecular complexity in GC, involving epitranscriptomic alterations, that provide a novel perspective on cancer development.

However, it should be taken into account that AGS and SNU638 were first chosen as models for this project because, together, they could provide a more insightful understanding of the heterogeneity of phenotypes found in GC when it comes to the expression of the reader proteins YTHDF1/2/3. Therefore, the variation observed in cell viability, or in the expression of protein levels in the two cell lines can also mean that different YTHDF3 targets exist in both cell lines. This could entail different aberrant expression of key genes involved in cell survival. It should be kept in mind that, different cancers may express diverse levels of these proteins, which can translate in unique reactions, while in some cases a higher expression of YTHDF3 is apparently related to a better prognosis as in gastrointestinal (Zhao *et al.,* 2020) or liver (Zhang *et al.,* 2019) cancers, and in breast cancer it is correlated to a poor prognosis (Liu *et al.,* 2019).

Even though it is a promising subject and the focus of recent studies, the involvement of reader proteins, especially YTHDF3, in cancer is yet to be fully understood. Thus far, the obtained results here presented suggest that m^{6}A RNA modifications might play a role in GC progression, suggesting that the YTHDF family, particularly YTHDF3 may be a possible biomarker of therapy response in GC. Clearly, these results are far from being fully informative on the exact function the YTHDF protein play in GC, yet they lay the way for a series of exciting questions on the effects of these proteins on GC that will need further consideration.

As the project, in which this thesis was included, is still ongoing other features regarding YTHDF3 will be further explored, and by combining the present approach with animal models and clinical samples we expect, in the future, to obtain a full picture on the function of the YTHDF proteins, and how they influence gene expression regulation in GC, thus creating new knowledge on the emerging field of post-transcriptional gene regulation.

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Appendix

	YTHDF1	YTHDF2	YTHDF3
Salivary gland	2*	-	2
Tongue	2	2	3
Stomach	2	2*	3
Small intestine	2	2	2
Colon	1*	2	3
Prostate	2	-	-
Testicle	2*	1	2
Breast	2	2	3
Liver	0	1	3
Kidney	2*	2	3
Adrenal gland	0	2	1
Lung	2*	3	3
Pancreas	2	2	3
Thymus	2	4	4
Gallbladder	2	2	1
Heart	2	0	1
Tonsils	2	-	4
Lymph node	0	2	3
Cerebellum	0	3	2*
Oesophagus	2	4	2
Spleen	0	4	2
Striated muscle tissue	0	0	0
Skin	0	2	2

Table legend:

0 – negative
1 – dim
2 – positive
3 – very positive
4 – extremely positive
- – inexistent core
* – nuclear