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Glycan- based nanotherapeutics for selective drug delivery in gastric cancer

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GLYCAN-BASED NANOTHERAPEUTICS FOR SELECTIVE DRUG DELIVERY IN GASTRIC CANCER

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Look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious.

Stephen Hawking

NOTA PRELIMINAR

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referência. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

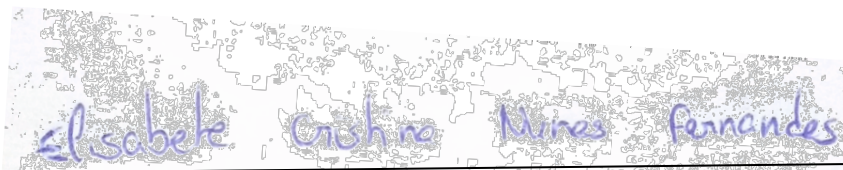
Os trabalhos de investigação desta tese foram realizados no Grupo de Patologia e Terapêutica Experimental do Centro de Investigação do Instituto Português de Oncologia do Porto, EPE e no Grupo de *Nanomedicine e Translation drug delivery* no Instituto Universitário de Ciências da Saúde (I3S)- Instituto Nacional de Ciências Biomédicas (INEB).

Declaro ainda que o presente trabalho foi financiado pela Fundação para a Ciência e a Tecnologia, pela atribuição de uma bolsa individual de doutoramento, com a referência SFRH/BD/103571/2014. A Fundação para a Ciência e a Tecnologia é co-financiada pelo Fundo Europeu de Desenvolvimento Regional da União Europeia, através do Programa Operacional Fatores de Competividade do Quadro de Referência Estratégico Nacional. Reconheço também o financiamento, pela Fundação para a Ciência e a Tecnologia, do Centro de Investigação do Instituto Português de Oncologia do Porto (PEst-OE/SAU/UI0776/201; CI-IPOP-29-2014; CI-IPOP-58-2015). Por último, declaro que o presente trabalho teve ainda o suporte financeiro do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

DECLARAÇÃO DE HONRA

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referência. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico. Mais ainda, o pedido de autorização para integração dos artigos “New trends in guided nanotherapies for digestive cancers: A systematic review” publicado na revista Journal of Controlled Release e do artigo “Glycoengineered nanoparticles enhance the delivery of 5-fluorouracil and paclitaxel to gastric cancer cells of high metastatic potential” na revista International Journal of Pharmaceutics encontram-se submentidos às respectivas revistas.

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Elisabete Cristina Nunes Fernandes

Em obediência ao disposto no nº 2 do Artigo 8º do Decreto-Lei nº 388/70, o autor declara que participou na conceção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redação dos trabalhos a seguir referenciados, estejam estes já publicados ou em fase de publicação e que integram a tese aqui apresentada:

1. **Fernandes E.***, Ferreira JA.*, Peixoto A., Lima L., Barroso S., Sarmento B. and Santos LL. New trends in guided nanotherapies for digestive cancers: A systematic review. *Journal of Controlled Release*. 2015. 209: p. 288-307. Impact Factor: 7.70, Q1. doi: 10.1016/j.jconrel.2015.05.003.
2. **Fernandes E.***, Sores J.*, Cotton S.* and Peixoto A.*, Ferreira D., Freitas R., Reis CA., Santos LL. and Ferreira JA. Gastroesophageal and colorectal cancers: Looking beyond classical serological biomarkers towards glycoproteomics-assisted precision oncology. *Molecular Aspects of Medicine*. 2019. Impact Factor: 10.24, Q1. (*Submitted*).
3. **Fernandes E.***, Ferreira D. *, Peixoto A., Freitas R., Relvas-Santos M., Palmeira C., Martins G., Barros A., Santos LL., Sarmento B., Ferreira JA. Glycoengineered nanoparticles enhance the delivery of 5-fluorouracil and paclitaxel to gastric cancer cells of high metastatic potential. *International Journal of Pharmaceutics*. 2019. Impact Factor: 4.21, Q1. doi: 10.1016/j.ijpharm.2019.118646.

* Equal contribution.

Para além dos trabalhos científicos anteriormente mencionados e que integram esta tese, durante o período de doutoramento, o autor da presente tese participou nos seguintes artigos complementares:

- Neves M.*, Azevedo R.*, Lima L., Oliveira Ml., Peixoto A., Ferreira D., Soares J., **Fernandes E.**, Gaiteiro C., Palmeira C., Cotton S., Mereiter S., Campos D., Afonso LP., Ribeiro R., Fraga A., Tavares A., Mansinho H., Monteiro E., Videira P., Freitas PP., Reis CA., Santos LL., Dieguez L., Ferreira JA., Exploring sialyl-Tn expression in microfluidic-isolated circulating tumour cells: a novel biomarker and an analytical tool for precision oncology applications. *New Biotechnology*. 2018. 49:77-78. Impact Factor: 3.73, Q1. doi: 10.1016/j.nbt.2018.09.004.
- Azevedo R.*, Soares J.*, Gaiteiro C., Peixoto A., Lima L., Ferreira D., Relvas-Santos M., **Fernandes E.**, Ana Tavares., Cotton S., Daniel-da-Silva AL., Santos LL., Vitorino R., FAmado F., Ferreira JA. Glycan affinity magnetic nanoplatfroms for urinary glycobiomarkers discovery in bladder cancer. 2018. 184:347-355. *Talanta*. Impact Factor: 4.16, Q1. doi: 10.1016/j.talanta.2018.03.028.
- Ferreira, JA., Magalhaes A., Gomes J., Peixoto A., Gaiteiro C., **Fernandes E.**, Santos LL. and Reis CA. Protein glycosylation in gastric and colorectal cancers: Toward cancer detection and targeted therapeutics. *Cancer Letters*, 2017. 387: p. 32-45. Impact Factor: 6.49, Q1. doi: 10.1016/j.canlet.2016.01.044.
- Lima L.*, Neves M.*, Oliveira Ml., Dieguez L., Freitas R., Azevedo R., Gaiteiro C., Soares J., Ferreira D., Peixoto A., **Fernandes E.**, Montezuma D., Tavares A., Ribeiro R., Castro A., Oliveira M., Fraga A., Reis CA., Santos LL., Ferreira JA. Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. *Urologic Oncology: Seminars and Original Investigations*. 2017. 35(12):675. Impact Factor: 3.77, Q1. doi:10.1016/j.urolonc.2017.08.012.

- Azevedo R.*, Peixoto A.*, Gaiteiro C, **Fernandes E.**, Neves M., Lima L., Santos LL., Ferreira JA. Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology? *Oncotarget*. 2017. 8(53):91734-91764. Impact Factor: 5.17, Q1. doi:10.18632/oncotarget.19433.
- Cotton S.*, Azevedo R.*, Gaiteiro C., Ferreira D., Lima L., Peixoto A., **Fernandes E.**, Neves M., Neves D., Amaro T., Cruz R., Tavares A., Rangel M., Silva AMN., Santos LL, Ferreira JA. Targeted O-glycoproteomics explored increased sialylation and identified MUC16 as a poor prognosis biomarker in advanced stage bladder tumours. *Molecular Oncology*. 2017. 11(8):895-912. Impact Factor: 5.37, Q1. doi:10.1002/1878-0261.12035.
- Peixoto A.*, **Fernandes E.***, Gaiteiro C.*, Lima L., Azevedo R., Soares J., Cotton S., Parreira B., Neves M., Amaro T., Tavares A., Teixeira F., Palmeira C., Rangel M., Silva AMN., Reis CA, Lúcio Lara Santos, Maria José Oliveira, José Alexandre Ferreira. Hypoxia enhances the malignant nature of bladder cancer cells and concomitantly antagonizes protein O-glycosylation extension. *Oncotarget*. 2016. 7(39):63138-63157.. Impact Factor: 5.17, Q1. doi:10.18632/oncotarget.11257.
- **Fernandes E. ***, Peixoto A. *, Neves M., Afonso LP., Santos LL., Ferreira JA. Humoral response against sialyl-Le(a) glycosylated protein species in esophageal cancer: Insights for immunoproteomic studies. 2015. 36(23):2902-7. *Electrophoresis*. Impact Factor: 3.16, Q2. doi: 10.1002/elps.201500270

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RESUMO

O Cancro Gástrico (CG) é a terceira causa de morte por cancro em todo o mundo, fazendo desta uma das doenças mais dispendiosas para os sistemas de saúde. Atualmente, as abordagens terapêuticas defrontam obstáculos significativos devido à falta de modelos moleculares essenciais para a estratificação dos doentes. Bem como, a necessidade da introdução alternativas mais eficazes à quimioterapia convencional e com menos efeitos adversos. Mais ainda, a considerável heterogeneidade molecular do GC ostenta um desafio não apenas para a subtipagem molecular, mas também para a identificação de biomarcadores dirigidos. Assim, a introdução de novas abordagens terapêuticas tem sido limitada neste modelo tumoral, incluindo as novas nanoterapias direcionada, capazes de fornecer seletivamente agentes de quimioterapias às células tumorais, com poucos efeitos secundários e maior eficiência terapêutica.

Alterações na glicosilação de proteínas traduzem-se na sobre-expressão de sialil-Tn (STn) e sialil-Lewis A (SLe^A), à superfície das células tumorais de CG, desempenhando um papel relevante na progressão e disseminação de células tumorais em diferentes níveis (migração, invasão, metástases e a evasão ao sistema imunitário. Estes glicanos são utilizados em testes serológicos, para o *follow-up* e prognóstico não invasivo dos doentes de CG e de outros modelos tumorais, no entanto apresentam baixa sensibilidade e especificidade para o diagnóstico. Também existe ainda muita controvérsia no uso destes testes para estratificar os doentes. Contudo, o facto destes glicanos, STn e SLe^A, serem expressos à superfície das células e serem específicos de células tumorais torna-os potenciais alvos para terapias dirigidas. Nomeadamente, podem ser utilizados no melhoramento de nanoterapias e torná-las mais eficazes para o CG

Assim, neste trabalho serão estabelecidos os princípios necessário para tal, nomeadamente: i) avaliar a expressão de STn e SLe^A no contexto clínico de CG, com o objetivo de estabelecer modelos moleculares capazes de estratificar os doentes; ii) estabelecer as bases moleculares para nanoterapias direcionados para estes glicanos com agentes de

quimioterapia encapsulados para células tumorais mais agressivas; iii) Caracterização glicoproteômica do GC, nomeadamente a caracterização de assinatura moleculares com o objetivo de obter alvos mais precisos.

O primeiro objetivo foi a caracterização do STn e do SLe^A no GC, em contexto clínico. Foi avaliada a adição destes a modelos moleculares, para estratificar doentes, em que também se incluiu a expressão de E-caderina (fenótipo epitelial em comparação com fenótipos mais mesenquimais) e Ki-67 (marcador de índice de proliferação), usados atualmente na clínica. Para tal, foi utilizada uma série de tecidos tumorais de CG (270 casos) representativos de todos os estadios da doença. Observou-se que a expressão do STn e do SLe^A, apresentava um padrão difuso na grande maioria dos casos de tumores gástricos e metástases. Foi também possível observar que o STn estava associado com metástases ganglionares regionais enquanto que o SLe^A estava associado com a metástases à distância, demonstrando duas diferentes vias de disseminação da doença, que requerem mais estudos neste sentido. Mais ainda, denotou-se a presença de um subgrupo de tumores proliferativos de natureza com melhor prognóstico. Ao contrário de outro subgrupo que apresentava baixa expressão de E-caderina e Ki-67, isto é, um grupo com baixa taxa de proliferação com natureza mesenquimal, com pior prognóstico. Foi também discriminado um terceiro grupo, de natureza híbrida, caracterizado por níveis variáveis de E-caderina ou Ki-67 e sobre-expressões de STn e SLe^A, com um prognóstico muito semelhante ao grupo pouco proliferativo de cariz mesenquimal, sugerindo que a perda de características epiteliais e proliferativas associadas a uma sobre-expressão de marcadores de glicosilação podem desempenhar um relevante papel no prognóstico de CG. Estas características foram avaliadas dentro das classificações clínico-patológicas clássicas (TMN, Lauren vs Borrmann).com o objetivo de avaliar o seu potencial preditivo. Posto isto, este trabalho propõe a integração de um painel molecular composto por biomarcadores clássicos e de glicosilação em sistemas de classificação clinicopatológica atualmente usados na clínica, prevendo que esta integração aperfeiçoe a estratificação dos doentes. Mais ainda, a expressão aberrante à superfície das células de

STn e de SLe^A, poderá revelar alvos para tumores com características mais agressivas. Assim, a segunda parte do trabalho explorou a presença do antígeno SLe^A, como alvo, em células de GC com alto potencial metastático. Posto isto, foi desenvolvida uma glico-nanopartícula funcionalizada com um anticorpo de SLe^A, para uma entrega controlada de fármacos quimioterapêuticos (5-fluorouracil e paclitaxel), dirigida a células de GC que expressam SLe^A. Foi possível observar que esta abordagem terapêutica permitiu a entrega seletiva de agentes citostáticos, *in vitro* e *ex vivo*. Notavelmente, observou-se que estas glyco-nanopartículas demonstraram ter baixa afinidade para tecidos saudáveis, demonstrando que esta abordagem terapêutica pode reduzir a toxicidade apresentada pelos fármacos quimioterapêuticos. São agora necessários estudos pré-clínicos *in vivo* para implementação desta terapia na clínica. Por último, foi avaliado a expressão de glicoproteínas com afinidade para a E-selectina, em diferentes linhas celulares de cancro gástrico, prevendo a identificação de características moleculares associadas ao desenvolvimento de metástases, mediada pela adesão de células cancerígenas a células epiteliais. Usando-se várias abordagens de imunoafinidade glicômicas e glicoproteômicas com base em bioinformática, foi possível identificar um conjunto de 22 glicoproteínas comuns a diferentes linhas celulares. Mais ainda, identificou-se a nucleolina (NCL), uma proteína nuclear em células saudáveis, na membrana de células cancerígenas gástricas e que expressam SLe^A. A avaliação da nucleolina em tumores gástricos primários e metastáticos assim como numa ampla variedade de tecidos saudáveis demonstrou que a expressão incorreta de nucleolina à superfície das células pode ser usado como alvo de células que apresentam maior potencial metastático. Esta é a primeira vez que é descrito, a expressão de SLe^A numa proteína preferencialmente nuclear, estabelecendo um novo paradigma para a descoberta de biomarcadores. A informação glicoproteômica gerada pode agora ser usada como ponto de partida para melhorar a especificidade da terapêutica direcionada baseada em SLe^A.

Em suma, a sobre-expressão de SLe^A e STn acompanhada pela sobre-expressão de E-caderina e Ki-67, permite a discriminação de um grupo de

doentes, com tumores agressivos com pior prognóstico. Além disso, estes antígenos permitiram o desenvolvimento de uma nanoterapia guiada, que poderá beneficiar estes doentes. Sendo estes marcadores translacionais a outros modelos tumorais, a terapia desenvolvida neste trabalho revela elevado potencial no desenvolvimento da medicina de precisão.

ABSTRACT

Gastric cancer (GC) is one of the most significant health care burdens worldwide, being the third leading cause of cancer-related death. Current therapeutic management faces significant hurdles associated with the lack of accurate molecular models for patient stratification. There is also an unmet need for more effective and less-toxic alternatives to conventional chemotherapy. Finally, the significant molecular heterogeneity presented by GC poses a significant challenge not only to molecular subtyping but also to the identification of potentially targetable biomarkers. This has limited the introduction of novel therapeutics approaches, including emerging targeted nanotherapies, capable of selectively delivering therapeutic agents to cancer cells with limited off-target effects and higher therapeutic efficiency.

Alterations in protein glycosylation translated by the overexpression of the sialyl-Tn (STn) and sialyl-Lewis A (SLe^A) at the cell-surface are common features of GC cells, playing relevant functional roles in cancer progression and dissemination at different levels (migrations, invasion, metastasis and immune escape). These glycans have been broadly explored in serological tests for non-invasive follow-up and prognostication in GC and other tumours but lack the necessary sensitivity and specificity for diagnosis. Some controversy also remains regarding their utility for patient stratification. However, the cell-surface and cancer-associated nature of STn and SLe^A holds great potential for targeted therapeutics. Moreover, these glycans may constitute an important strategy towards more effective nanotherapies against GC.

As such, this thesis was devoted to setting the necessary milestones towards this end, which included: i) providing a clinical context for STn and SLe^A expressions in GC, envisaging molecular models for patients stratification; ii) setting the molecular foundations for glycan-targeted nanovehicles for delivering chemotherapy agents to more aggressive cancer cells; iii) Glycoproteomics characterization of GC envisaging unique molecular fingerprints precise cancer targeting. The first objective has addressed based on the comprehensive characterization of the clinical

context underlying STn and SLe^A expressions in GC. The goal was to pursue the incorporation of glycans in a molecular model for patient stratification that also included E-cadherin (epithelial-like vs mesenchymal-like phenotypes) and Ki-67 (proliferation index) expressions, already used in the clinics. A broad series of tissue sections of GC (270 cases) representative of all stages of the disease was used towards this end. STn and SLe^A antigens were diffusely expressed throughout the vast majority of GC and metastasis, in accordance with their functional role. Notably, STn was associated with lymph node metastasis, whereas SLe^A was associated with distant metastasis, denoting different glycan-mediated disease dissemination routes that warrant future research. On the other hand, E-cadherin and Ki-67 overexpressions defined a subgroup of proliferative tumours of epithelial-like nature and better prognosis. Contrastingly, low E-cadherin and Ki-67 highlighted a low-proliferative group, potentially of mesenchymal-like nature, facing worst prognosis. A third group, of hybrid nature was also identified, characterized by variable levels of either E-cadherin or Ki-67 but significant STn and SLe^A overexpressions. Tumours of hybrid character showed a strikingly lower survival, very similar to the low-proliferation mesenchymal-like group, suggesting that the loss of either proliferative or epithelial traits and marked altered glycosylation may play a role driving worst prognosis. These molecular features were successfully applied to improve the predictive potential of classical clinicopathological classifications (TMN, Lauren vs Borrmann). As such, this work proposes the integration of a molecular panel composed by glyco- and classical biomarkers into broadly used clinicopathological classification systems, foreseeing improved patient stratification. Moreover, aberrant cell surface expression of STn and SLe^A may provide means to selectively target more aggressive tumours. Based on this rationale, the second part of the work exploited the SLe^A antigen to target GC cells of high metastatic potential. SLe^A-targeted glycoengineered nanoparticles (NPs) were developed to deliver chemotherapy to metastatic SLe^A positive GC cells. Glycan-based NPs enabled the controlled release of cytotoxic drugs (5-fluorouracil and paclitaxel), selectively targeting SLe^A cells *in vitro* and *ex vivo*. Notably,

glycan-targeted nanoparticles showed limited affinity to healthy tissues, suggesting that this approach may reduce the off-target toxicity presented by chemotherapy. Pre-clinical studies *in vivo* are now required foreseeing the clinical implementation of this solution. Finally, different gastric cancer cell models were interrogated for SLe^A-expressing glycoproteins with affinity for E-selectin, foreseeing the identification of molecular features associated with metastasis development by mediating cancer cells adhesion to epithelial cells. Using a combination of immunoaffinity glycomics and glycoproteomics assays backed by bioinformatics it was possible to identify a subset of 22 glycoproteins common to different cell lines that potentially play a critical role in this process. Moreover, we have identified nucleolin (NCL), typically a nuclear protein in healthy cells, at the membrane of gastric cancer cells and expressing SLe^A. The screening of gastric cancer primary and metastatic lesions and a wide array of healthy tissues further demonstrated that the miss-localization of nuclear NCL to the cell surface may be used to selectively target cancer cells presenting higher metastatic potential translated by higher affinity to E-selectin. To our knowledge, this is the first report describing SLe^A in a protein preferentially found in the nucleus, setting a new paradigm for cancer biomarker discovery. The generated glycoproteomics information may now be used as starting point to improve the specificity of SLe^A-based targeted therapeutics.

Altogether, this work establishes important milestones facing the molecular characterization of GC, while defining particularly aggressive tumour phenotypes driving decreased patients' cancer-specific survival. It highlights the potential of altered glycosylation in this context, while providing the molecular foundations to guide nanotherapeutics to more aggressive cancer cells. Given the pancarcinomic nature of aberrant glycosylation, the translation of this solution to other models may also be envisaged.

GRAPHICAL ABSTRACT

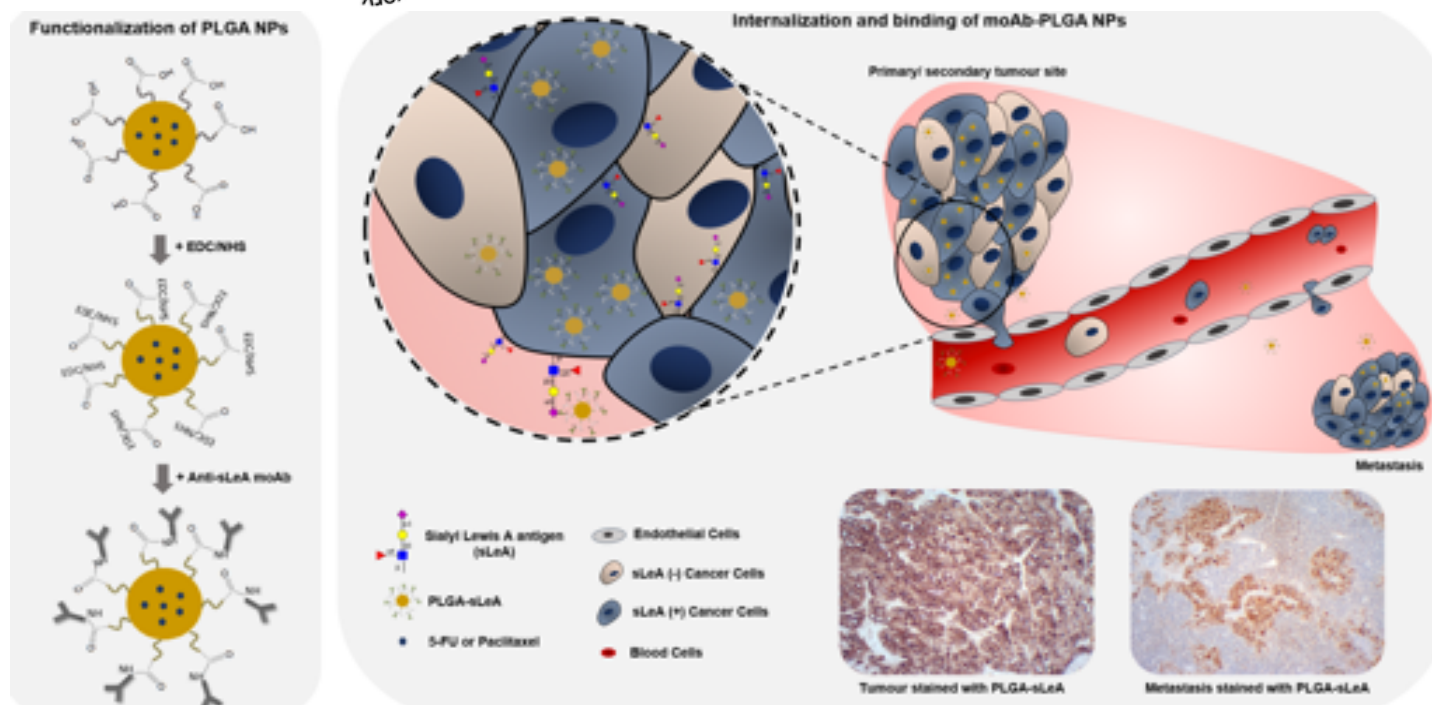
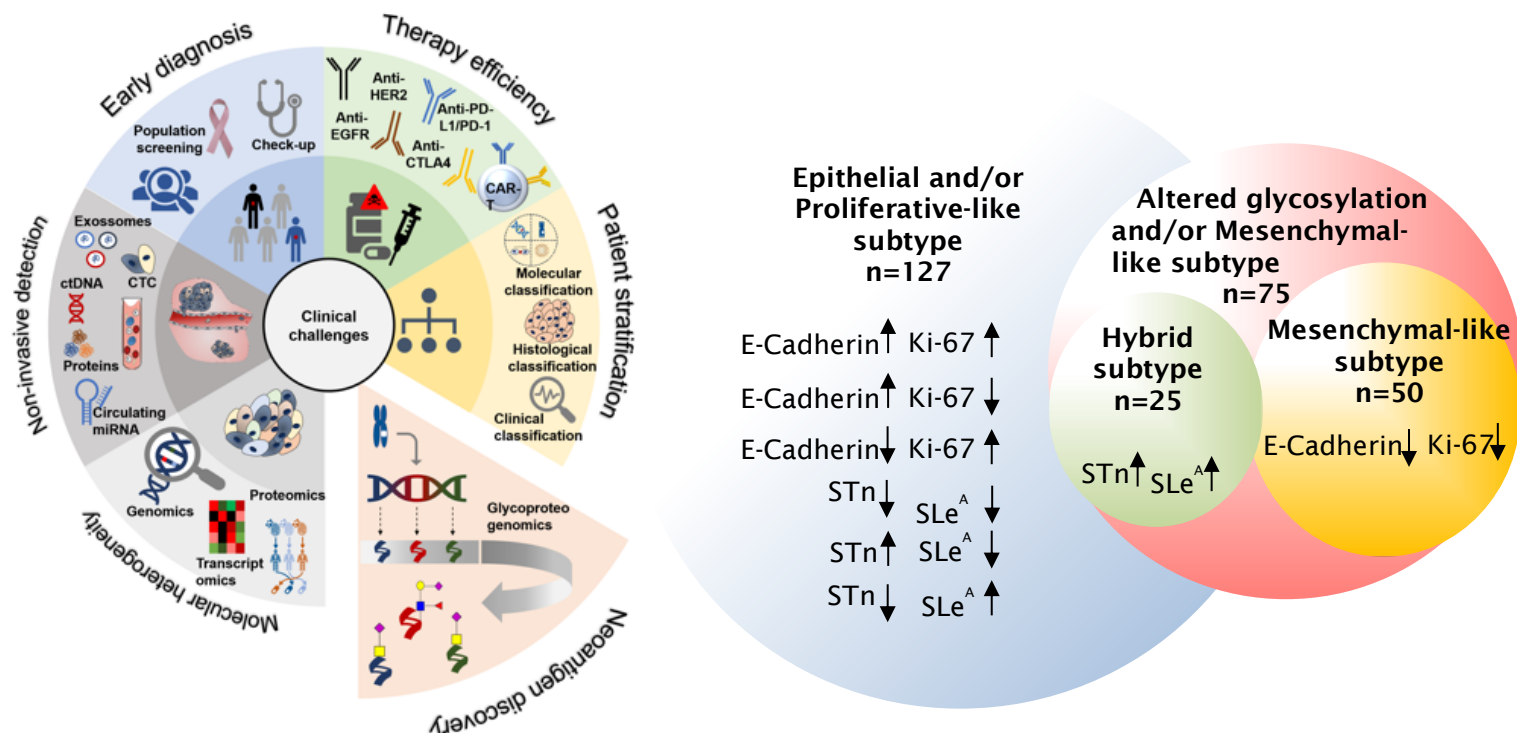


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ABBREVIATIONS & ACRONYMS

5-FU: 5-Fluorurcil Analyses

ACRG: Asian Cancer Research Group

AFP: Alphafetoprotein

CagA: Cytoxon-Associated Gene A

CAR: Chimeric Antigen Receptor

CDH1: E-cadherin

CEA: Carcinoembrionic Antigen

CIN: Chromossomal Instability

CSS: Cancer Specific Survival

DC: Dentrific Cells

DFS: Disease Free Survival

EBV: Epstein-Barr Virus

EGC: Early Gastric Cancer

EMT: Epithelial-to-Mesenchymal Transition

EPR: Enhanced Permeability and Retention

GAPPS: Gastric Adenocarcinoma And Proximal Polyposis Of The Stomach

GC: Gastric Cancer

GIST: Gastrointestinal Stromal Tumours

GS: Genomically Stable

HCC: Hepatocelular Carcinoma

HDGG: Herditary Diffuse Gastric Cancer

IGCA: International Gastric Cancer Associated

IM: Intestinal Metaplasia

MALT: Mucosa-Associated Lymphoid Tissue

MSI: Microsatelite Instable Tumours

OS: Overall Survival

PD: Poorly Differentiated

PFS: Pogression Free Survival

PLGA: Poly(lactic-co-glycolic Acid)

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-

PTM: Post-tranlation Modification

PTX: Paclitaxel
SLe^A: sialyl lewis a
SLe^x: sialyl lewis x
SPSS: Statistical Package for Social Sciences
SRC: Signet-ring cell Carcinoma
STn: sialyl-Tn
TCGA: The cancer Genoma Atlas
TMN: Tumour Node Metastasis
VacA: Vacuolating Cytotoxin A
VEGF: Vascular Endothelial Growth Factor
WD: Well Differentiated
WHO: World Health Organization

THESIS OUTLINE

This thesis addresses two main concerns associated with gastric cancer (GC) management, the absence of reliable biomarker panels for easy patient stratification in clinical settings and targetable molecules for constructing innovative, less toxic and more effective therapeutics.

It hypothesises that altered protein glycosylation translated by the overexpression of cancer-associated sialyl-Tn (STn) and sialyl-Lewis (SLe^A) antigens maybe used, together with other classical biomarkers, to improve GC patients' stratification. The fact that these glycan alterations occur at the cell-surface and present a restricted expression pattern in most healthy tissues also suggests potential for targeted therapeutics. In another dimension of this work, biocompatible nanoencapsulation systems are regarded as ideal and innovative strategies for delivering chemotherapy agents to cancer cells, while reducing the toxicity and improving their therapeutic efficacy. Exploitation altered glycosylation to target these nanoconstructs to more aggressive cancer cells is regarded possible and may constitute an important milestone towards more effective therapeutic approaches.

Addressing these hypotheses, the present document is divided into four main sections: i) **The introduction**, providing an overview on GC, its clinicopathological and molecular classification, therapeutic approaches, main clinical challenges and glycan-based opportunities facing therapeutic intervention. It also includes a broad comprehensive review on the functional role and clinical potential of STn and SLe^A in gastroesophageal and colorectal cancers, setting critical milestones for intervention. Namely, it focuses on the pressing need to progress towards targeted glycoproteomics settings focusing on STn and SLe^A envisaging cancer-specific molecular signatures. This is expected to overcome limitations associated the sensitivity and specificity for cancer, due to their restricted expression in few healthy tissues and some subpopulations of blood cells. This goal is considered critical for the generalization of glycan-based therapeutics and, ultimately, improved cancer control. The last part of the

introduction comprehends a systematic review on recent advances on targeted nanotherapies for digestive cancer, with emphasis on GC. Focus is set on the enormous therapeutic potential of these solutions for cancer treatment and outstanding challenges facing clinical translation, including the identification of clinically relevant and targetable biomarkers. The exploitation of glycans towards this end is emphasised. The second section, the **Aims and Scopes**, builds on the introduction to summarize the main clinical challenges faced by GC patients, setting the opportunities for improvement provided by glycans and nanotherapies in this context. It also defines the main objectives of the thesis and provides an overview on the adopted research strategy. The third section, **the Results**, is mostly of innovative dimension. It describes the main contribution of the thesis to advances in the state-of-the-art. It is formed by three chapters corresponding to two unpublished (**chapters 1 and 3**) and one published manuscript (**chapter 2**). **Chapter 1** addresses the clinical relevance of STn and SLe^A in GC, aiming to improve patient prognosis. It also aims to set the molecular rationale for targeted intervention. This includes the development of glycan-targeted nanotherapies (**Chapter 2**) and glycoproteomics studies for precise mapping of cancer-specific glycoprotein signatures (**Chapter 3**). Therefore, **Chapter 3** is expected to set the necessary molecular rationale for refining further patient molecular subtyping and improve targeted therapeutics, with emphasis on the solutions proposed in **Chapter 2**. **Concluding Remarks and Future Perspectives** envisaging true improvements in GC patient's management.

INTRODUCTION

The information provided in this section is based in the following publication:

- **Fernandes E.***, Ferreira JA.*, Peixoto A., Lima L., Barroso S., Sarmento B. and Santos LL. New trends in guided nanotherapies for digestive cancers: A systematic review. *Journal of Controlled Release*, 2015. 209: p. 288-307. Impact Factor: 7.70, Q1. doi: 10.1016/j.jconrel.2015.05.003
- **Fernandes E.***, Sores J.* , Cotton S.* , Peixoto A.* , Ferreira D., Freitas R., Reis CA, Santos LL. and Ferreira JA. Gastroesophageal and colorectal cancers: Looking beyond classical serological biomarkers towards glycoproteomics-assisted precision oncology. 2019. *(submitted)*

Overview of Gastric Cancer, Clinical Management Challenges and Opportunities

Epidemiology

Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third leading cause of death from cancer, with an estimated 1,0 million new cases and 627,000 deaths in 2018 [1]. Asia, Eastern Europe and South America are endemic regions, while the United States and Western Europe report the lowest incidence rates [1-3]. The predominant risk factor for GC is *Helicobacter pylori* (*H.pylori*) induced gastritis, accounting for approximately 60% of cases [4]. Namely, *H.pylori* comprises several virulence factors capable of inducing the neoplastic transformation of gastric epithelium, including: i) CagA induction of proliferation, cytoskeletal reorganization and invasion of epithelial cells, ii) CagA induction of pro-inflammatory cytokines and oncoproteins, iii) methylation of tumor suppressor genes as *CDH1*, *TFF2*, *RUNX3*, *FLNc*, *HAND1*, *THBD*, *p41ARC*, *HRASLs* and *LOX*, and iv) chronic inflammation-induced reactive oxygen and nitrogen species causing tissue and DNA damage [2, 5, 6]. However, the progression of gastric carcinogenesis has been shown to be related with host characteristics, such as genetic polymorphisms of pro-inflammatory interleukins [7, 8] and molecules involved in adhesion of the bacteria [9-11], as well as genetic variations in virulence factors of the bacteria such as cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*) [7]. In addition, age, tobacco and alcohol consumption, poor diet, pernicious anemia and other chronic gastric diseases also constitute established promoters of stomach carcinogenesis [12-17].

Currently, the implementation of population screening and intervention programs in developed countries has been contributing to *H. pylori* infection eradication, ultimately leading to the declining of gastric cancer incidence [18]. However, total number of cases is expected to rise as a result of

population ageing and GC still constitutes a significant healthcare burden [18, 19].

Gastric Cancer Pathophysiology and classification systems

The most common type of GC is adenocarcinoma (90% of cases), followed by lymphoma or gastrointestinal stromal tumors (10% of cases). More rare GC subtypes include mucosa-associated lymphoid tissue (MALT)-lymphomas, gastrointestinal stromal tumours (GIST), carcinoid tumors, and leiomyosarcomas [20]. Most GC cases are sporadic in nature, but approximately 10% display familial clustering and only 1–3% of these are thought to be hereditary. Hereditary syndromes comprise hereditary diffuse gastric cancer (HDGC) [21–23] and the recently described gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) [24, 25].

The tumour-node-metastasis (TNM) staging system has long been the standardized benchmark for classifying patients with GC, defining prognosis and determining the best treatment approaches considering the tumor invasion depth or T stage, regional lymph node counts and the presence of distant metastasis [26, 27]. Moreover, T stage and the number of metastatic lymph nodes are the most important prognostic factors for curative gastric cancer surgery [27]. Recently, an updated AJCC 8th TNM staging system was proposed based on the results of the International Gastric Cancer Association (IGCA) staging project, in which 25 411 eligible GC cases were collected retrospectively from 59 institutions in 15 countries [28]. Specifically, the AJCC 8th TNM staging system represents advancement in pN category, staging homogeneity, discrimination power, prognostication and reproducibility for prediction of GC prognosis. However, this classification has many pitfalls since the operator dependence and heterogeneity of data bias clinical decision [29].

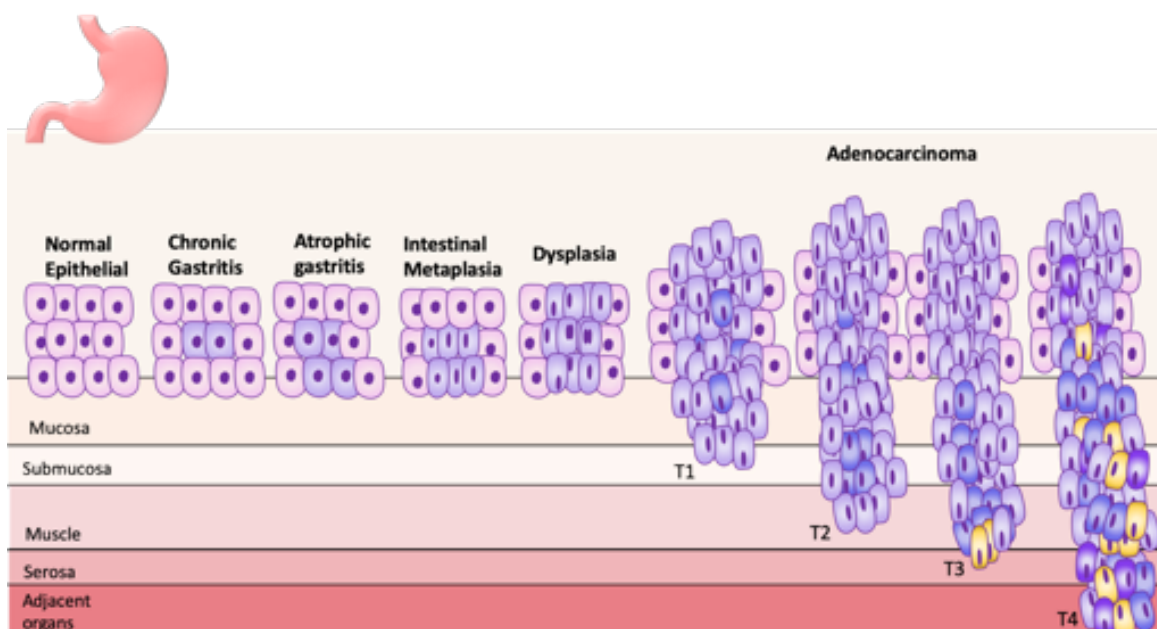


Figure 1. A summary of gastric carcinogenesis and GC T stage from TNM staging system. Cells alteration, due to environmental risks and host characteristics, through carcinogenesis from normal cells to adenocarcinoma. Regarding T stage of tumour T1, is based on invasion to gastric submucosa; T2, Tumour penetrating muscle; T3, Tumor invades serosa and T4 comprise tumour that invades adjacent organs.

During the last 50 years, the histological classification of gastric carcinoma has been largely based on Lauren's criteria [30], in which GC is classified according to two major histological subtypes, namely intestinal type and diffuse type adenocarcinoma. Of note, these correspond to the differentiated and undifferentiated types described by Nakamura et al [31]. Lauren's classification, although dated, is still widespread due to its immediacy and simplicity but it has been shown to be inadequate facing recent chemotherapeutic and surgical approaches. Furthermore, the prognostic relevance of Lauren's classification is still controversial. Some studies failed to find any correlation between this classification and patient's outcome [32], others demonstrated its prognostic significance [33] and others described Lauren's classification as an independent prognostic factor [34]. Notwithstanding, intestinal tumours are very cohesive, commonly arranged in tubular or glandular formations, and are often associated with intestinal metaplasia [30]. This type of gastric cancer affects the gastric antrum and is associated with lymphatic and vascular invasion, being

commonly associated with good prognosis [34, 35]. In turn, diffuse gastric tumours are less cohesive, mainly due to E-cadherin dysregulation, and infiltrate the stroma as single or small groups of cells [34]. It usually affects the body of the stomach, eventually progressing to peritoneal dissemination. Moreover, diffuse gastric cancer is characterized by poor prognosis compared to the intestinal type [36]. Of note, the pathogenesis of both intestinal and diffuse gastric carcinomas involves DNA methylation, histone modifications and chromosome recombination [37, 38]. However, the intestinal type is more associated with environmental risk factors, whereas the diffuse type usually presents a genetic aetiology [39]. In this context, *H.pylori* infection is the most studied promoter of intestinal gastric cancer, promoting a neoplastic cascade of atrophic gastritis, intestinal metaplasia, dysplasia and ultimately adenocarcinoma [39, 40]. In turn, active gastritis is considered to be the major risk factor for diffuse gastric carcinoma, even though *H.pylori* infection can also lead to diffuse GC [41, 42]. In addition, a small percentage of gastric cancers exhibit a familial cluster associated with *CDH1* germline mutations, causing the hereditary diffuse gastric cancer syndrome [43]. Specifically, *CDH1* mutations lead to decreased expression of CDH1 (also known as E-cadherin), which decreases cell adhesion and activates multiple signal transduction pathways, leading to tumor invasion and metastasis [44]. In summary, the two proposed histological subtypes of GC present distinct clinical and molecular characteristics, which could provide the basis for individualized treatment. To meet this goal, some recent studies have been investigating individualized treatment guided by Lauren's pathological classification, which can be found thoroughly explored in recent reviews [45]. Moreover, the inclusion of molecular data to improve this classification has been attempted. Accordingly, diffuse and intestinal type gastric carcinomas were found to differ in their expression of apoptosis related proteins, namely Fas and Bcl-xl expression, bringing Lauren's classification one step closer to patients stratification [46].

The World Health Organization (WHO) histological classification for GC is also widely accepted and used. Moreover, it has been proven useful and necessary during the perioperative management of early gastric cancer (EGC) patients [47]. However, its impact on predicting lymph node metastasis and recurrence is not well studied. Using this classification, gastric adenocarcinomas are divided into papillary, tubular, mucinous, and signet-ring cell carcinoma (SRC) [48]. In addition, tubular adenocarcinoma is further graded into well-differentiated adenocarcinoma (WD), moderately-differentiated adenocarcinoma (MD), and poorly-differentiated adenocarcinoma (PD). This classification also integrates the Nakamura classification concepts by classifying tumour as differentiated and undifferentiated types. In this context, the former includes WD, MD, and papillary adenocarcinoma, and the latter includes PD, SRC, and mucinous adenocarcinoma. The impact of WHO classification on predicting lymph node metastasis and recurrence in EGC has been recently revised by Ji Fu Lai et al. [47]. Of note, tubular and papillary carcinomas roughly correspond to the intestinal type described by Lauren's, while signet-ring cell carcinoma would correspond to the diffuse type.

Based on the gross appearance of gastric carcinomas, the Borrmann's classification divides these tumours as type I for polypoid growth, type II for fungating growth, type III for ulcerating growth, and type IV for diffusely infiltrating growth, also referred to as linitis plastica in signet ring cell carcinoma when most of gastric wall is involved by infiltrating tumor cells. Bormann type IV tumours represent approximately 10% of advanced gastric carcinoma cases, comprising rapidly proliferating and poorly differentiated cells and extensive stromal fibrosis [49]. While informative, the macroscopic appearance of advanced tumours is not sufficiently accurate to provide pre-operative staging information; thereby, this information is often complemented with imageology techniques [50].

Of note, the previously mentioned GC classifications translate the histological and aetiological heterogeneity of tumours. However, they lack robust molecular characterization and the identification of dysregulated

pathways and candidate drivers of distinct classes of gastric cancer. To meet this need, a comprehensive molecular evaluation of 295 primary gastric adenocarcinomas as part of The Cancer Genome Atlas (TCGA) project gave rise to a molecular classification dividing GC into four subtypes. Namely, tumours positive for Epstein-Barr virus (EBV), displaying frequent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, PD-L1 and PD-L2; microsatellite unstable tumours (MSI), showing elevated mutation rates, including mutations of genes encoding targetable oncogenic signalling proteins; genomically stable tumours (GS), which are enriched for the diffuse histological variant and mutations of RHOA and CDH1 or fusions involving RHO-family GTPase-activating proteins; and tumours with chromosomal instability (CIN), showing marked aneuploidy and focal amplification of receptor tyrosine kinases (HER2, EGFR, FGFR e MET) [51]. The identification of these molecular milestones has provided a roadmap for targeted therapeutics, while allowing patient stratification. Based on this classification a recent prediction model successfully stratified patients by survival and adjuvant chemotherapy outcomes. Namely, the EBV subtype was associated with the best prognosis, while GS subtype was associated with the worst prognosis. Patients with MSI and CIN subtypes had poorer overall survival than those with EBV subtype but better overall survival than those with GS subtype. Furthermore, patients with the CIN subtype experienced the greatest benefit from adjuvant chemotherapy, while GS patients were the least benefited [52]. Overall, this classification system allows the stratification of patients better served by adjuvant chemotherapy.

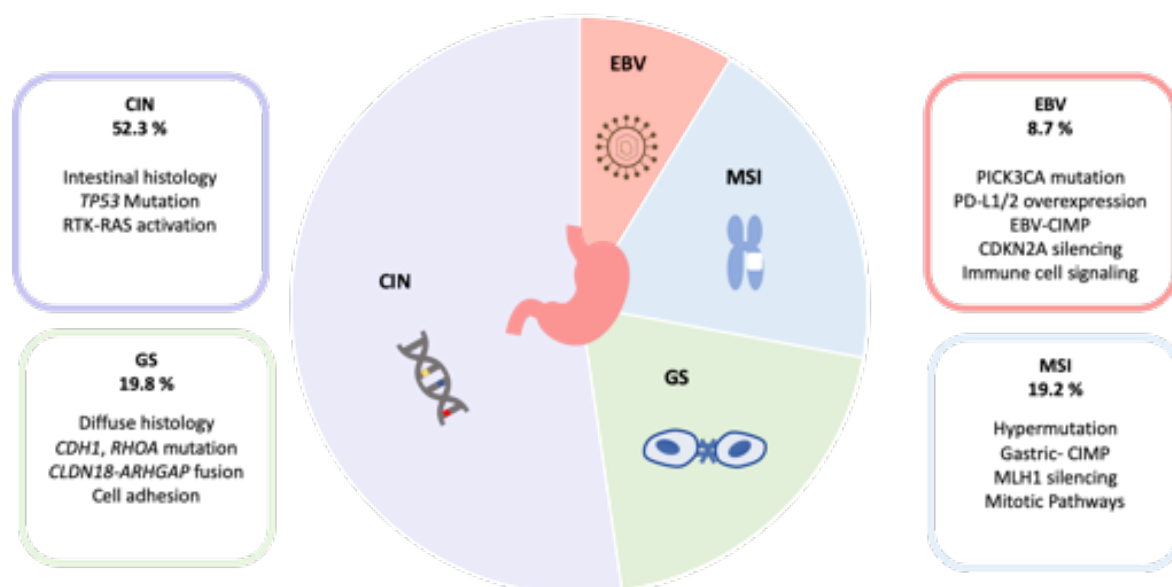


Figure 2. Schematic representation of comprehensive molecular characterization of gastric carcinoma. Percentage list distribution of the major features of the four molecular subtypes of gastric cancer molecular subtypes Epstein-Barr virus (EBV), microsatellite instability (MSI), genomically stable tumours (GS) and chromosomal instability (CIN).

Another classification was proposed by Asian Cancer Research Group (ACRG), dividing GC into subtypes according to distinct patterns of molecular alterations, disease progression and prognosis [53]. Accordingly, GC was divided into four groups, including microsatellite instability (MSI), microsatellite stability and epithelial-to-mesenchymal transition (MSS/EMT), microsatellite stability and presence of TP53 (MSS/TP53+) or microsatellite stability and absence of TP53 (MSS/TP53-). Particularly, the MSI subtype was associated with hypermutation, including in the genes *KRAS* (23.3%), *ALK* (16.3%) and *ARID1A* (44.2%). Moreover, it was associated with good prognosis and low recurrence rates. Of note, most cases correspond to the Lauren's intestinal subtype. The MSS/EMT group shows loss of *CDH1* expression and is characterised by poor prognosis and high recurrence rates. Most cases correspond to the Lauren's diffuse type. Finally, the MSS/TP53 groups present intermediate prognosis and recurrence rates compared to the other subgroups. The MSS/TP53+ was frequently associated with EBV infection, presenting high rates of *PC*, *ARID1A*, *KRAS*, *PIK3CA*,

SMAD4 mutation. In turn, the MSS/TP53- subtype demonstrated amplification of *HER2*, *EGFR*, *MYC*, *CCNE1*, *CCND1*, *MDM2*, *ROBO2*, *GATA6* and *MYC* both at the DNA and mRNA level [53]. This classification system provides a consistent and unified framework for further clinical and preclinical translational research.

Finally, the ‘Singapore-Duke’ classification identifies three major subtypes of GC with distinct biological properties and sensitivity to chemotherapy or targeted agents. Namely, the proliferative, metabolic and mesenchymal subtypes. Accordingly, the mesenchymal type was strongly associated with the Lauren’s diffuse type and poorly differentiated GC, being characterized by epithelial-to-mesenchymal transition and stem cell pathways activation. Moreover, cell lines of the mesenchymal subtype were particularly sensitive to phosphatidylinositol 3-kinase-AKT-mTOR inhibitors. The proliferative subtype was strongly associated with the Lauren’s intestinal type, presenting oncogenic pathways activation, such as E2F, MYC and RAS, and cell cycle genes deregulation. Cancer cells of the metabolic subtype were more sensitive to 5-fluorouracil (5-FU) than other cell lines, probably due to significantly lower expression of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) involved in 5-FU resistance mechanisms [54]. Analysis of cancer-specific survival showed no significant survival difference among the three subtypes. However, these findings might be useful in selecting specific treatment approaches for GC patients.

Overall, the above-mentioned classifications attempt to stratify gastric tumours according to their histological natures and more recently to their molecular characteristics. However, few studies try to correlate their finds with previous classification systems, which translates in a conundrum of unconnected possible stratifications. Notwithstanding, the need for targeted therapeutics and patient stratification has accelerated the discovery of molecular targets and the investigation of chemotherapy response of tumours within this classification systems. Future studies should focus on including proteomic information into the molecular panels already

established as well as update currently used clinical classifications to improve therapeutic decision.

Main Gastric Cancer Management Challenges

Even though GC incidence has fallen in developed countries in recent years, advances in disease treatment have been limited, mostly due to its above-mentioned genetic complexity and heterogeneity.

Surgery remains the mainstay treatment for early-stage GC, and the only possible curative opportunity. Endoscopic resection may also be an option for well differentiated, confined to the mucosa and non-ulcerated early-stage tumours [55]. In patients with localized disease, surgery alone or multi-modal therapeutic schemes are pondered according with tumour stage. Notwithstanding, perioperative epirubicin, cisplatin and 5-fluorouracil (5-FU) (ECF) has been shown to significantly improve 5-year survival compared to surgery alone (36.3% vs 23%, respectively) in patients with resectable GC [56]. Adjuvant chemo(radio)therapy and postoperative chemotherapy may also result in a survival benefit for patients with resectable localised disease [57-59]. However, more than half of patients relapse locally or with distant metastases [60]. Furthermore, to improve early-stage patients' outcomes, improved methods of staging incorporating prognosis and nodal risk are warranted.

Approximately two-thirds of patients diagnosed with GC present locally advanced or metastatic disease, with a median survival not exciding 10 months [61] and 5-year overall survival (OS) rates reaching about 15% [62]. Standard treatment for advanced stage GC is chemotherapy regimens based on fluoropyrimidine, platinum, taxanes, topoisomerase inhibitors or anthracyclines. However, the standard of care is poorly defined, with no preferred first- or second-line regimens. Moreover, optimal combinations are patient dependent, based on comorbidities, performance status (PS) and organ function. Notwithstanding, chemotherapy provides survival and quality of life benefits compared to best supportive care (BSC) alone [55, 63-65]. Preferred first-line regimens include cisplatin combined with a

fluoropyrimidine (fluorouracil or capecitabine) or cisplatin and a fluoropyrimidine combined with a third agent, which is typically epirubicin or docetaxel. Second-line therapy normally includes single-agent irinotecan or taxane (docetaxel or paclitaxel). The addition of taxanes offers a benefit in overall survival compared to previous options; however, with considerable increase in toxicity [66, 67]. Of note, half of advanced stage GC patients treated with standard chemotherapy progress to metastatic disease within 5 years and many die prematurely from adverse side-effects as neuropathy, cardiopathy or immunosuppression [68, 69], urging for targeted therapeutic options.

Advanced gastric tumours frequently present receptor tyrosine kinase (RTK)/RAS amplifications (e.g. fibroblast growth factor receptor 2 [FGFR2], *ERBB2/HER2*, *EGFR* and *MET*). As such, approximately 37% of patients could be potentially treated by RTK/RAS-targeted therapies [70]. Accordingly, advanced HER2⁺ gastric cancer patients are managed using trastuzumab (anti-p185Her-2/neu) combined with a fluoropyrimidine (5-FU or capecitabine) and cisplatin, resulting in improved disease-free survival (DFS) and overall survival (OS) [55, 71, 72]. In addition, overexpression of vascular endothelial growth factor (VEGF) has been associated with advanced-stage disease, poorer outcomes and bad prognosis in gastric cancer [73]. Hence, tumour angiogenesis inhibitors as bevacizumab (anti-VEGF-A) and ramucirumab (anti-VEGFR) were brought into therapeutic schemes in combination with chemotherapy for first-line treatment of advanced GC. Ramucirumab has been demonstrated effective while bevacizumab did not improve overall survival of advanced patients [74-76]. These findings might be due to the fact that ramucirumab blocks VEGFR-2, the primary receptor for VEGF-A, rather than blocking the ligand itself. Also, aberrant activation of Hepatocyte growth factor receptor/c-Met signalling pathway is an independent prognostic factor for worse outcomes in GC, suggesting the therapeutic potential of c-Met [77]. In this context, c-Met inhibitors, as rilotumumab, ficlatuzumab and onartuzumab are under extensive clinical trials [77]. However, the crosstalk between c-Met and other RTKs, which is

responsible c-Met inhibitors resistance, suggests that monotherapy is not an effective therapeutic approach but novel combinatory strategies should be designed. The coexistence of genetic alterations within tumors may also affect the response to c-Met targeted agents. These findings reinforce the need for stratification biomarkers capable of identifying patient subgroups capable of benefiting from targeted therapeutics.

Overall, treatment options for patients with locally advanced and metastatic gastric cancers are limited, and new therapies to improve quality of life and prolong survival are needed.

Glycosylation in gastric cancer carcinogenesis

Glycosylation is one of the most common post-translation modifications (PTM) of secreted and membrane-bound proteins, as well as a common substitution in lipids at the cell surface [78]. Glycans are not codified by genetic information. However, sugar chains arise from the coordinated action of nucleotide sugar transporters, glycosyltransferases and glycosidases within the endoplasmic reticulum (ER) and the Golgi Apparatus (GA). This highly regulated biosynthetic process gives rise to the two major classes of protein-bound glycans, namely *N*-glycans in Asparagine (Asn) residues of polypeptide chains and *O*-glycans in serine (Ser) or threonine (Thr) moieties [79, 80]. The carbohydrate structures of these main glycan classes can be further elongated, trimmed or chemically modified based on glycosyltransferase specificity and location within the cell. Briefly, the simplest form of protein *O*-glycosylation is the monosaccharide Tn antigen, synthesized by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases (ppGalNAc-Ts) in the ER. This structure is generally extended by T-synthase in a COSMC dependent manner to originate the disaccharide Thomsen-Friedenreich or T antigen. Tn and T antigens can be sialylated by sialyltransferases, forming the sialyl-Tn (STn), sialyl-T and disialyl-T antigens, preventing subsequent processing or elongation. The T antigen may be extended to form core structures 2-4, which are precursors for a vast array of more extended oligosaccharides and terminal structures [81].

In turn, *N*-glycans biosynthesis starts in the ER with the oligosaccharide transferase complex (OSTase)-mediated addition of a precursor glycan to Asn residues in Asn-X-Ser/Thr peptides (X stands for any amino acid except proline). This forms a common *N*-glycan core structure that is further processed in the ER and GA by multiple glycosyltransferases and glycosidases to yield the three major *N*-glycan types: oligomannose, complex, and hybrid. Hybrid and complex *N*-glycans may be further substituted with *N*-acetylglucosamine (GlcNAc) residues through the action of GlcNAcT-III (GnT-III) to originate bisecting core structures, stopping any additional elongation or processing. Highly branched *N*-glycans may be generated by the action of several GlcNAc transferases (GnT-IV, -V, -VI) and be further extended by the addition of galactose, poly-*N*-acetylglucosamine, sialic acid, and fucose residues. Both, *O*- and *N*-glycans frequently exhibit Lewis (Le) blood group related antigens (Le^A, Le^x, Le^B and Le^y) and corresponding sialylated structures or ABO(H) blood group determinants as terminal epitopes [80]. As a consequence of the highly diverse glycan repertoire, glycans are involved in several important intra- and extracellular functions, as growth factor receptor regulation, cell adhesion and motility, immune recognition, endothelium attachment, among others [82, 83].

Aberrant glycosylation patterns are common features of solid tumours [83, 84], including GC [85], being detected even in pre-malignant lesions such as intestinal metaplasia (IM) [86]. Several glycosylation alterations have been described in GC, with emphasis on E-cadherin regulation. E-cadherin is a transmembrane protein with key roles in cell-cell interactions and adhesion, contributing to the homeostasis of tissue architecture [87]. As such, downregulation or inactivation of E-cadherin-mediated cell-cell adhesion takes place in initial steps of GC carcinogenesis. Specifically, E-cadherin is decreased or absent in pre-invasive lesions of hereditary diffuse gastric cancer (caused by CDH1 germline mutations). Contrastingly, alterations in E-cadherin expression occur predominantly at later stages of intestinal subtype GC progression [88, 89]. Moreover, E-cadherin dysfunction has been extensively associated with poor clinical outcomes in GC patients

[90, 91]. The aberrant E-cadherin expression in GC has been shown to result from genetic, epigenetic, and post-translational changes (70% of cases) [90]. These alterations include *CDH1* point mutations [92, 93], *CDH1* epigenetic silencing through promoter hyper-methylation [44, 94, 95], microRNAs regulation of the E-cadherin transcriptional repressors zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2 [96-98] as well as E-cadherin *N*-glycosylation. Specifically, the extracellular domain of E-cadherin has four potential *N*-glycosylation sites (Asn554, Asn566, Asn618 and Asn633) with key roles in protein folding, expression, and biological functions [99, 100]. Namely, E-cadherin lacking *N*-glycosylation at Asn-633 was recognized as a substrate of ERAD, being retro-translocated from the ER lumen to the cytoplasm for ubiquitination and subsequent degradation as a consequence of misfolding [100]. Also, non-glycosylated E-cadherin was modified with immature high mannose type *N*-glycans, suggesting that it could not depart to the Golgi apparatus, compromising protein trafficking [100]. Moreover, the interaction between E-cadherin and p120 catenin, which regulates E-cadherin endocytosis and stability at the cell surface, is also regulated by E-cadherin *N*-glycosylation. Hence, GnT-III-derived bisecting GlcNAc structures stabilize E-cadherin at the cell membrane, precluding E-cadherin endocytosis, leading to a stable E-cadherin/catenin adhesion complex and promoting cell-cell aggregation. This E-cadherin glycoform promotes increased intercellular adhesion and a downregulation of intracellular signalling pathways involved in cell motility and EMT, supporting its contribution to tumor suppression [101-103]. Contrastingly, the modification of E-cadherin with β 1,6 GlcNAc branched structures catalysed by GnT-V led to a destabilization of E-cadherin, inducing a disorganization of the cadherin/catenin complex and ultimately impairing cell-cell aggregation of GC cells [104]. Accordingly, in GC, E-cadherin immunostaining is not always translated by the absence of expression (as seen upon epigenetic or structural alterations), but frequently shows a redistribution from the cell membrane into the cytoplasm. Apart from E-cadherin regulation, GnT-V is frequently upregulated in GC where it

contributes to increased cancer cell invasion and metastasis through β -1,6GlcNAc branched *N*-glycans overexpression in other adhesion molecules [105-107].

Integrins are also highly glycosylated proteins, playing key roles in cell-ECM interactions. Thereby, changes in integrin glycosylation have tremendous impact on cell migration, signalling transduction and metastasis [108]. In the context of GC, specific modification of $\alpha 3\beta 1$ integrin with $\beta 1,6$ GlcNAc-branched *N*-glycans promotes increased cell migration on ECM components [109]. Moreover, GnT-III activity inhibited GnT-V-induced cell migration by countering the effect of GnT-V-mediated glycosylation [109]. Accordingly, GnT-III inhibits cell motility and adhesion by steadying E-cadherin at the cell surface and stabilizing cell junctions as well as by downregulating integrin interaction with ECM components.

Gastric cancer cells also commonly overexpress sialylated antigens, specially sialylated lewis blood group antigens (SLe^A and SLe^x) and STn, which are significantly correlated with patients' poor prognosis, while being mostly absent from healthy tissues [110, 111]. Namely, in GC, STn overexpression was mostly associated with decreased overall survival, venous and lymphatic invasion, depth of invasion, tumour stage, and lymph node metastasis [110-112]. In turn, high expression of dimeric SLe^x correlated with venous invasion and decreased patient survival [113], while SLe^A correlated with increased depth of invasion and tumour size [114]. Furthermore, SLe^{A/x} are E-selectin ligands in vascular endothelial cells, facilitating tumour cell intravasation into primary tumour blood vessels and circulating tumour cells arrest in distant locations during hematogenous metastasis [115-117]. On another note, SLe^{A/x} are specific ligands of sialic acid binding adhesin (SabA) that promotes *H. pylori* adhesion to the inflamed gastric mucosa [118]. Moreover, SLe^x expression in the gastric epithelium is further induced during persistent *H. pylori* infection, creating a chronic inflammation state capable of establishing precursor neoplastic lesions of the gastric epithelium [119]. These findings shed a light on the prognostic value of sialylated glycans in GC and suggest an active role in

tumour cell aggressive phenotypes. For instance, GC cells overexpressing ST3GAL4 and consequently SLe^x showed enhanced invasive characteristics both *in vitro* and in *in vivo* CAM model. Moreover, these glycoengineered models displayed increased c-Met RTK activation as well as phosphorylation of its downstream targets FAK, Src proteins, Cdc42, Rac1 and RhoA GTPases to increase invasion [120]. In turn, SLe^A was found to be a common PTM of human epidermal growth factor receptor 2 (ErbB2), determinately affecting its expression levels and possibly its activating dimerization [121]. Furthermore, an association between the expression of ERBB2 and FUT3, a central gene in SLe^A biosynthesis, was disclosed in GC patients, further highlighting the crosstalk between ErbB2 and SLe^A expression [121]. In this context, sialyl lewis antigens actively contribute to cancer associated inflammation.

Regarding STn, ST6GalNAc-I is the major enzyme controlling the expression of this cancer-associated antigen in gastrointestinal tissues [122, 123]. Through STn overexpression in GC cells, ST6GalNAc-I modulates processes like cell cycle, apoptosis, actin cytoskeleton dynamics, adhesion and motility on ECM components, cell-cell aggregation, invasion and metastasis [124-126]. Noteworthy, glycoengineered cancer cells overexpressing STn resulted in less cohesive tumor xenografts, leading to decreased mice survival. Furthermore, truncation of *O*-glycans induced activation of EGFR and ErbB2 receptors as well as transcriptomic changes in GC cells. The disclosed top activated genes were further validated in gastric tumors, revealing that *SRPX2* and *RUNX1* are concomitantly overexpressed in GC tissues and its expression is associated with patients' poor-survival, highlighting their prognosis potential for clinical practice [126]. Furthermore, STn overexpression in GC cells promoted increasingly chemoresistant phenotypes by driving the intracellular accumulation of galectin-3 and decreasing its interaction with cell surface glycan receptors [127]. These findings highlight the need for the development of novel strategies to target galectin-3 and/or ST6GalNAc-I. Furthermore, *O*-glycoproteomic characterization of GC cell lines has revealed CD44 and

GalNAc-T5 STn glycoforms as potential biomarkers in GC, highlighting the cancer specific nature of STn and the biomarkers potential of glycoproteins [128]. Altogether, these findings emphasise the role of STn in the acquisition of malignant features related to increased migration, invasion and chemoresistance of GC cells.

Overall, the above-mentioned rationale associated with a restricted expression pattern in healthy tissues, makes these glycans a good starting point for developing targeted therapeutics.

Glycan-based opportunities facing gastric cancer clinical challenges

Gastric cancer is mostly asymptomatic until it progresses to advanced stages [129]. As such, early detection using effective screening approaches is of major importance for GC patient's management. Notwithstanding, GC is a complex disease, displaying significant molecular heterogeneity, which makes biomarker discovery an intricate task. Although many biomarkers for GC have been reported, including carbohydrate antigen CA125, alpha-fetoprotein (AFP), SLE, BCA-225, hCG and pepsinogen I/II, carcinoembryonic antigen (CEA), CA72-4 and CA19-9 are the most frequently used biomarkers in clinical practice [130]. Particularly, both STn and SLe^A antigens are often present in proteins that are secreted or shed from tumours into circulation and can be detected using the CA72-4 (for the STn antigen) and CA19-9 (for the SLe^A antigen) serological assays. The CA19.9 test is broadly used to monitor recurrence and response to therapy in GC [131]. Furthermore, preoperative positivity for CA19-9 is an independent risk factor for hematogenous recurrence, which should be considered when advising adjuvant chemotherapy after GC surgery [132]. However, there is limited application for this serological marker in GC due to false positives related with SLe^A expression in benign lesions and false negatives occurring in individuals with Lewis (a-b-) genotype [133]. In turn, CA72-4 is a clinically useful marker in diagnosis, evaluation of chemotherapy efficacy, and prognosis of patients with GC peritoneal dissemination [134]. However, CA72-4 is also detected in the serum of patients displaying benign lesions,

indicating that routine screening of CA72-4 levels for diagnosing GC in asymptomatic patients may be ineffective due to low sensitivity and low positive predictive value [135, 136]. As such, the reduced specificity and sensitivity of these serological assays for early detection of cancer is driving a search for novel biomarkers facing the correct clinical challenges. Novel glycomics and glycoproteomics strategies combining molecular information from glycan and peptide moieties holds tremendous potential for biomarker discovery, opening an avenue for designing highly specific targeted therapeutics [137]. In other models, specific glycoforms of aberrantly expressed glycoproteins provided accurate biomarkers for early disease detection. Specifically, the fucosylated AFP is a current FDA-approved serological marker for early detection of hepatocellular carcinoma (HCC), reaching its peak levels during liver cirrhosis before the neoplastic transformation to HCC [138, 139]. In GC, an STn expressing glycoform of plasminogen, a glycoprotein involved in ECM remodelling during *H. pylori* chronic infection of the gastric mucosa, was found in the serum of patients with gastritis and intestinal metaplasia (IM) [136]. These findings provide evidence of serum proteins displaying abnormal O-glycosylation in patients with precursor lesions of GC and include a panel of putative targets for the non-invasive clinical diagnosis of individuals with gastritis and IM.

In line with this, the cancer specific and cell surface nature of aberrant glycosylation constitutes an attractive target for guided therapeutics. Looking at highly context-specific glycosignatures as attractive targets for personalized medicine, several glycan-based therapeutic strategies and immunotherapies have emerged. Namely, inhibitors of glycosyltransferases catalytic activity [140] and glycoengineered antibodies capable of inducing antibody-dependent cellular cytotoxicity [141]. Furthermore, glycan-based antibodies may be used to guide nanotherapies [142, 143] or improve the cancer specificity of chimeric antigen receptors (CAR) of genetically modified T cells [144]. On another note, tumour specific glycoliposomes can be used to prime dendritic cells (DC) through DC-SIGN engagement to stimulate tumour-specific CD4⁺ and CD8⁺ T cell anti-tumour responses,

constituting a promising glycan-based immunotherapy [145]. Finally, anticancer multicomponent glycoconjugate vaccines, based on glycan antigens coupled to T-cell peptide epitopes or immunostimulant epitopes, have been demonstrated effective in circumventing cancer immunotolerance [146, 147], providing an appealing option for the much-awaited development of new glycan-based therapeutic agents. Notwithstanding, GC remains an orphan disease regarding the introduction of novel targeted therapeutics into clinical practice.

Targeted nanotherapeutics for advanced stage gastric cancer patient mannagement

Conventional therapeutic approaches of surgical resection and adjuvant chemotherapy have so far demonstrated little success in the management of advanced stage GC patients. Specifically, in non-metastatic advanced GC (>T1N0), the best available systemic therapy combinations only yield a median progression-free survival (PFS) time of 5 to 7 months and a median OS in the range of 8 to 11 months [148]. Moreover, conventional chemotherapy commonly acts as a selective pressure for more aggressive and undifferentiated clones capable of recapitulating the tumour heterogeneity and quickly progress to metastasis [149]. In addition, systemic chemotherapy comprises significantly debilitating side-effects, limiting the acceptable therapeutic dosages and compromising overall outcomes [150]. Also, abnormal tumour vasculatures and significant stromal barriers often result in the delivery of sub-optimal drug concentrations to the tumour bulk, further contributing to chemotherapy resistance [151]. Based on this rational, novel targeted approaches capable of improving drug delivery while decreasing drug toxicity are warranted. In this context, nanoencapsulation of chemotherapeutic agents by biocompatible molecules holds great potential to exceed some of these limitations and add therapeutic specificity. Namely, co-encapsulation of different drugs in the same nanocarrier can provide multifaceted approaches without increasing toxicity [152]. Moreover, nanotherapies

preferentially build-up in tumour sites compared to healthy tissues through a mechanism known as enhanced permeability and retention (EPR) effect in which the leaky tumour vasculature favours nanocarriers intravasation [153]. Drug-loaded nanoparticles may accumulate in tumor tissues solely due to the EPR effect (passive nanocarriers) or may actively target cancer cells through functionalization, further increasing therapeutic specificity [154]. The most common homing molecules include monoclonal antibodies, antibody fragments, peptides and proteins, carbohydrates and aptamers, all of which significantly enhancing target cell recognition, cell uptake and tissue microdistribution in comparison to non-guided nanotherapeutics [143]. Several review work extensive explore the progress and challenges towards targeted delivery of cancer therapeutics [154-156]. In the present work, biocompatible poly(lactic-co-glycolic acid) (PLGA) nanoparticles were produced to encapsulate chemotherapeutic payloads. Nanoparticles surface was functionalized with an anti-SLe^A monoclonal antibody to increase cancer cell homing based on the cancer specific nature of aberrant glycosylation. Furthermore, it is anticipated that this may constitute an important milestone to establish improved glycan-based targeted therapeutics against gastric cancer.

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Gastroesophageal and colorectal cancers: Looking beyond classical serological biomarkers towards glycoproteomics-assisted precision oncology

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Abstract

Oesophageal (OC), gastric (GC) and colorectal (CRC) cancers are amongst the digestive track tumours with higher incidence and mortality due to significant molecular heterogeneity. This constitutes a major challenge for patients' management at different levels, including non-invasive detection of the disease, prognostication, therapy selection, patient's follow-up and the introduction of improved and safer therapeutics. Nevertheless, important milestones have been accomplished pursuing the goal of molecular-based precision oncology. Over the past five years, high-throughput technologies have been used to interrogate tumours of distinct clinicopathological natures, generating large-scale biological datasets (*e.g.* genomics, transcriptomics, proteomics). As a result, GC and CRC molecular subtypes have been established to assist patient stratification in clinical settings. However, such molecular panels still require refinement and are yet to provide targetable biomarkers. In parallel, outstanding advances have been made regarding targeted therapeutics and immunotherapy, paving the way for improved patient care; nevertheless, important milestones towards treatment personalization and reduced off-target effects are also to be accomplished. Exploiting the cancer glycoproteome for unique molecular fingerprints generated by dramatic alterations in protein glycosylation may provide the necessary molecular rationale towards this end. Therefore, this review presents functional and clinical evidences supporting a reinvestigation of classical serological glycan biomarkers such as sialyl-Tn (STn) and sialyl-Lewis A (SLe^A) antigens from a tumour glycoproteomics perspective. We anticipate that these glycobiomarkers that have served us well for non-invasive cancer prognostication may still hold value for patients' management in precision oncology settings.

Introduction

Oesophageal (OC), gastric (GC) and colorectal (CRC) cancers are part of the top ten most prevalent and deadliest tumours worldwide [1]. Despite representing a diversified array of diseases with distinct aetiologies and molecular backgrounds, these tumours share comparable management problems [2]. These include poor prognosis due to late diagnosis and the lack of efficient therapeutics to avoid disease progression, dissemination and metastases. Early detection based on large-scale screening of symptomatic and asymptomatic populations remains a complex task, relying almost exclusively on upper digestive tract endoscopy and colonoscopy. The absence of reliable non-invasive molecular tools for cancer detection has significantly delayed the generalization of life-saving interventions to a wider population (**Figure 1**). Moreover, patient management is further aggravated by the molecular heterogeneity presented by gastroesophageal and colorectal tumours, even between lesions of apparently similar histological nature [3-5] (**Figure 1**). Such aspects impede accurate patient stratification and lead to the adoption of ineffective therapeutic schemes that may be accompanied by severe toxicity, ultimately delaying or impairing more effective interventions. The identification of patients at higher risk of developing metastasis has also been particularly challenging, requiring a more profound knowledge of the intricate molecular networks established by cancer cells and the tumour stroma (**Figure 1**). In fact, these aspects are critical for inducing cell migration and invasion, supporting the formation of pre-metastatic niches and circulating tumour cells that dictate metastatic spread [6]. Another key challenge is therapeutic management. For most OC, GC and CRC lesions, first line therapy remains surgery and chemotherapy/radiotherapy [7, 8]. However, many subpopulations of cancer cells are endowed with chemotherapy resistance, leading to tumour relapse and unfavourable outcomes [9, 10]. The introduction of antibody-based targeted therapeutics with trastuzumab (anti-HER2), ramucirumab (anti-VEGFR2), and cetuximab (anti-EGFR) have increased patient survival in some subgroups, as highlighted by recent reviews [11, 12]. However, antibody-based targeted therapeutics efficiency may be considered modest, requiring refinements in the election of targeted groups and improvements in antibody-ligand recognition. The

recent introduction of immune check-point inhibitors targeting PD-1/PD-L1 and CTL4 has constituted an exciting milestone in the field of immunotherapy, with yet conflicting results regarding patient's survival [13-15]. Again, the existence of robust molecular models to predict patients better served by these approaches remains to be established, hindering true conclusions about therapeutic efficacy (**Figure 1**). It is also consensual that the multifactorial and dynamic molecular nature of gastroesophageal and colorectal tumours may most likely benefit from combination therapies supported by real-time assessment of response and rapid adaptation of therapeutic schemes, which is yet to be accomplished.

The comprehensive interrogation of gastroesophageal and colorectal tumours using genomics and transcriptomics has already translated into predictive molecular models for GC and CRC, which will decisively shape future clinical practice towards precision oncology [16-19] (**Figure 1**). Notably, OC has been so far mostly a neglected neoplasia regarding these objectives. In addition, genomics is also being used to guide proteomics studies in GC and CRC envisaging targetable biomarkers and patient personalization [17, 20, 21]. This constitutes the foundations for oncoproteogenomics that couples mass spectrometry (MS) approaches with high-throughput next-generation sequencing (NGS) technologies to study the role of protein variants in biological processes and pathology, while identifying cancer neoantigens for therapy development. This big push for systems biology is expanding fast, being complemented with novel layers of molecular information translated by post-translational modifications (PTM), which are critical for biological systems regulation. While much of the research focus has been put on the role played by phosphorylation, functional glycoproteomics has now demonstrated that glycosylation is also critical for defining key oncogenic features such as cell motility, invasion, metastasis and immune escape [22]. Alterations in protein glycosylation also decisively contribute to the activation of relevant oncogenic pathways that sustain cell survival and proliferation [22]. Moreover, cancer associated changes in the composition, density and distribution of glycosites are responsible by generating unique molecular signatures at the cell-surface holding tremendous potential for targeted therapeutics against membrane glycoproteins [23].

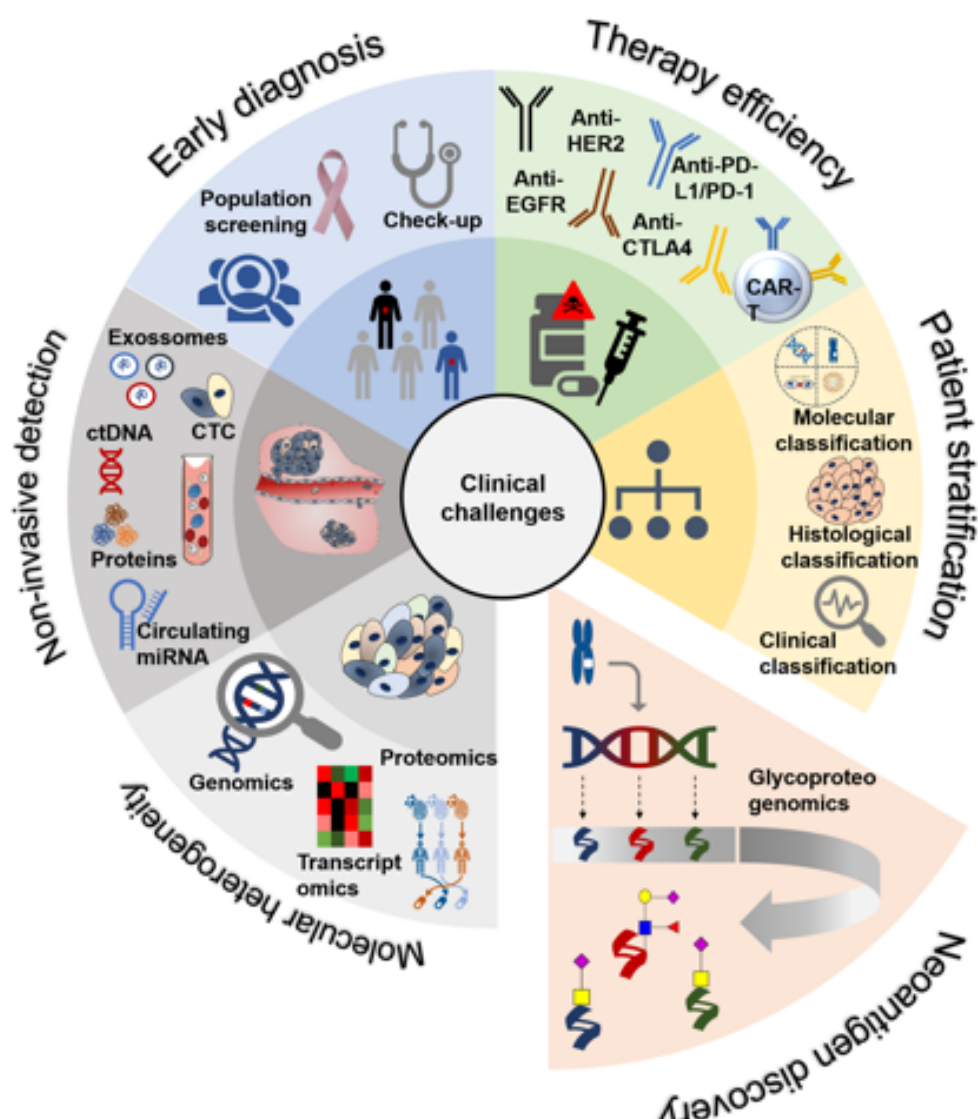


Figure 1. Gastroesophageal and Colorectal cancers main clinical challenges (inner circle), current clinical approaches (outer circle) and opportunities provided by neoantigen discovery. Current clinical challenges include: **i) Lack of robust early diagnosis tools**, requiring population screening and timely medical check-ups, especially for population subsets presenting known risk factors (poor diet; *H. pylori* infection; family history, age, gender, pre-neoplastic lesions; etc). The absence of molecular biomarkers with the necessary specificity and sensitivity to assist in this matter remains a tremendous obstacle for early cancer detection; **ii) Need for patient stratification**, mostly achieved based on the clinicopathological classification of the lesions and, most recently, moving towards the incorporation of molecular biomarkers; **iii) Therapy selection and efficacy**, currently based on clinicopathological features but rapidly evolving towards molecular-assisted settings capable of aiding therapy personalization and early definition of responders. Therapeutic management has been based on surgery, chemo and/or radiotherapy, encompassing severe toxicity and limited efficacy, particularly for advanced disease stages. However, this paradigm has started

to change with the introduction of antibody-based targeted therapeutics against key oncogenic cell surface receptors and immune-check point proteins such as PD-1, PD-L1 and CTLA4. CART-T immunotherapy is also amongst future promising approaches; **iv) non-invasive detection**, necessary for real-time monitoring of disease status and evolution throughout the course of disease. The field of liquid biopsies has tremendously evolved with the evaluation of circulating tumour DNA/miRNAs, proteins, micro and nanovesicles (exosomes and others) and, more recently, the study of circulating tumour cells (CTCs). The evaluation of these biomarkers in bodily fluids has improved prognostications and helped refining therapeutic selection, evaluating responses, establishing the risk of metastasis development and the detection of radiologically occult micrometastasis; **v) molecular heterogeneity** is also a critical clinical challenge. This aspect has been a major obstacle towards effective molecular-assisted oncology and the introduction of targeted therapeutics. Nevertheless, the field has experienced significant advances with next generation sequencing, which generated a significant amount of genomics and transcriptomics data that has been used to propose gastric and colorectal cancer molecular subtypes. Cancer proteomics characterization has also contributed to the identification of relevant biomarkers; however, with yet limited clinical translation; **vi) cancer neoantigens discovery** also represents a critical objective and a daunting challenge. It will be crucial for the identification of cancer-specific fingerprints capable of guiding therapeutic decision and designing effective targeted therapies and immunotherapy with very limited off-targeted effects. The comprehensive integration of genomics, transcriptomics and proteomics as well as information on post-translational modifications, with emphasis on glycosylation, will be of key importance for the identification of relevant protein functional nodes and targetable biomarkers at the cell-surface.

Two of the most studied glycans in OC, GC and CRC are the sialyl-Tn (STn) antigen, a short-chain glycan resulting from a premature stop in protein O-glycosylation, and the sialyl-Lewis A (SLe^A) antigen, a terminal glycoepitope of carbohydrate chains in glycoproteins and glycolipids. These glycoepitopes are absent or very strictly expressed in healthy tissues, being significantly overexpressed in advanced stage digestive tract tumours where they are frequently associated with poor prognosis [24, 25]. STn and SLe^A antigens are widely used in clinical practice for non-invasive follow-up based on the CA72-4 (for STn) and CA19-9 (for SLe^A) tests; however, they lack the necessary specificity and sensitivity for diagnosis and accurate patient stratification [26]. Nevertheless, there is a relevant amount of information supporting their involvement in cell invasion and metastasis development, providing a relevant rationale for intervention [23]. Moreover, there are several pre-clinical and clinical studies involving targeted therapeutics and

immunotherapy focused on these glycans showing exciting results but also outstanding challenges towards clinical implementation. As such, herein we provide a comprehensive review on the functional implications and current clinical significance of STn and SLe^A in gastroesophageal and colorectal cancer. We also highlight the potential of revisiting these glycans from a glycoproteomics perspective and attempt to set critical research milestones for its integration in emerging panomics studies envisaging future exploitation in the context of precision medicine.

Protein Glycosylation in cancer: Structural Diversity and Biological Significance facing clinical applications

The extracellular domains of most membrane-anchored proteins are glycosylated, often at multiple sites, creating a dense network of carbohydrate chains at the cell surface termed glycocalyx. There is growing evidence that the nature of cell surface glycosylation decisively impacts on the structure and function of membrane proteins and consequently on cell homeostasis [27-29]. The cancer glycoproteome may change at different levels, including alterations in the density and distribution of glycosites and/or glycan chains structure [22, 23, 30]. The most common glycosylation alterations in cancer, including in digestive tract tumours, comprise: i) changes in the substitution patterns of fucose and sialic acid residues [31, 32]; ii) dramatic reduction in *O*-GalNAc glycans extension (PTM of Ser and Thr residues) through oversialylation [33-35]; and iii) alterations in branching, core fucosylation and sialylation of *N*-glycans (PTM of Asn) [36-39]; iv) alterations in the nature of terminal glycan chain epitopes, including Lewis blood group patterns [40, 41]. Aberrant extracellular glycans may act as signal transducers, regulating key intracellular pathways involved in cell proliferation and survival [22, 29]. Furthermore, glycosylation modifications may significantly impair the normal function of key proteins involved in cell-cell and cell-extracellular matrix adhesion such as integrins, cadherins and CD44 towards more motile and invasive phenotypes [23, 42]. The glycode also governs recognition by immune cell lectins, dictating the fate of immune response, often towards more cancer tolerogenic phenotypes [43, 44]. Even though the molecular and clinical contexts

driving changes in glycosylation remain, in many cases, unclear, it is quasi unanimous that the tumour microenvironment promotes glycophenotypes that favour disease progression and dissemination [22, 45, 46]. Accordingly, the tumour microenvironment-tumour cell crosstalk is rapidly translated by glycosylation changes at the tumour cell surface to drive stress adaptation or active escape [22, 47]. Overall, glycans integrate extra- and intracellular cues that are phenotypically reflected at the cell surface, being active players in malignancy or surrogate markers of cellular adaptation. As such, glycans are attractive targets for theragnostic applications, which will be comprehensively addressed later in this review. Focus will be set on the most widely studied and clinically explored glycans in gastroesophageal and colorectal tumours, the sialyl-Tn (STn) and the blood group related sialyl lewis A (SLe^A) antigens.

Over- or *de novo*- expression of STn (STn; NeuAc α 2-6GalNAc α 1-O-Ser/Thr) antigen results from significant alterations in protein O-GalNAc glycosylation impairing its full extension and maturation. O-GalNAc glycosylation is a tightly regulated tissue-dependent process in which the density and distribution of glycosites is dictated by the nature of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases (ppGalNAc-Ts) present in each biological milieu [48]. It starts with the addition of GalNAc residues to serine (Ser) or threonine (Thr) moieties by one of twenty possible ppGalNAc-Ts, originating the Tn antigen [49]. The Tn antigen may be further extended into different core glycan precursors which may give rise to more complex glycan structures terminated by fucosylation and sialylation [38, 50]. Therefore, it is not uncommon to observe O-GalNAc glycans with terminal sialylated lewis blood group determinants such as the SLe^A antigen, particularly in the malignant gastrointestinal and colorectal mucosa [41, 51]. On the other hand, the Tn antigen may be sialylated by ST6GalNAc-I or -II to form STn, which stops further glycan chain elongation and largely reduces the O-glycans repertoire at the cell surface [52] (**Figure 2**). Known mechanisms underlying increased STn levels include overexpression of sialyltransferase *ST6GALNAC1* [53] and mutation [54] or hypermethylation [55] of the *COSMC* gene, leading to a downregulation of COSMC, a major core 1 synthase (C1GALT1) chaperone. This impairs O-glycan extension beyond the Tn antigen, that may that become available

for sialylation by ST6GalNAc-I [56]. Golgi-to-endoplasmic reticulum relocation of polypeptide *N*-acetylgalactosamine-transferases (GalNAc-Ts) also drives Tn accumulation in cancer cells [57], ultimately favouring STn biosynthesis (**Figure 2**). Notably, the Tn and STn antigens have been classically associated to mucins due to their high density of glycosylation sites and, consequently, are frequently termed simple-mucin type *O*-GalNAc glycans [50]. Nevertheless, virtually all glycoproteins presenting extracellular motifs prone to be *O*-glycosylated may express these glycoepitopes [58]. However, STn expression in healthy tissues is normally restricted to poorly represented secretory cells [59]. It is also not expressed by most blood cells, with the exception of some lymphocyte populations [60]. Contrastingly, STn is expressed by more than 80% of human carcinomas and, in all cases, it associated with adverse outcomes and decreased overall patients survival [59], making it a potentially low risk biomarker for targeted therapeutics.

Cancer glycoconjugates at the cell-surface also frequently overexpress sialylated lewis-type blood group antigens such as SLe^A (Sia α 2,3Gal β 1,3(Fuc α 1,4)GlcNAc-R, R being a portion of a glycan chain attached to a protein or lipid) and its isomer SLe^x (Sia α 2,3Gal β 1,4 (Fuc α 1,3)GlcNAc as terminal epitopes of *O*-glycans, *N*-glycans, and glycolipids [61]. However, SLe^A has perhaps attracted more attention in the context of serological assays and cancer research because, contrarily to SLe^x, it is not expressed by blood cells. The biosynthesis of sialyl Lewis antigens is a complex process involving the coordinated expression of several glycosyltransferases, which might be different depending on the nature of the glycoconjugate carrying the antigen (**Figure 2**). Increased SLe^{A/x} levels in *O*-glycans have been mostly associated with C2GnT overexpression [62], while aberrant glycolipid SLe^{A/x} has been corelated with the activation of β 1,3-GlcNAc transferase [63]. The competition between the biosynthesis of type 1 (SLe^A) and type 2 (SLe^x) chains is tissue specific and is mostly controlled by the basal levels of β -1,3 and β -1,4-galactosyltransferases [64]. During malignant transformation, overexpression of sialyl lewis antigens is also derived from neo-synthesis or incomplete synthesis of more complex lewis structures. Namely, non-malignant epithelial cells of the digestive tract predominantly express di-sialyl Lewis A (disLe^A) antigens, presenting an additional *O*-6 linked sialic acid than SLe^A [65]. In healthy

epitheliums, this isoform interacts with inhibitory sialic acid-binding immunoglobulin-type lectins (Siglecs) in immune cells, contributing to maintain tissue homeostasis [66, 67]. Upon neoplastic transformation, epigenetic silencing of *ST6GALNAC6* due to histone deacetylation and DNA methylation leads to the expression of the short isoform SLe^A.

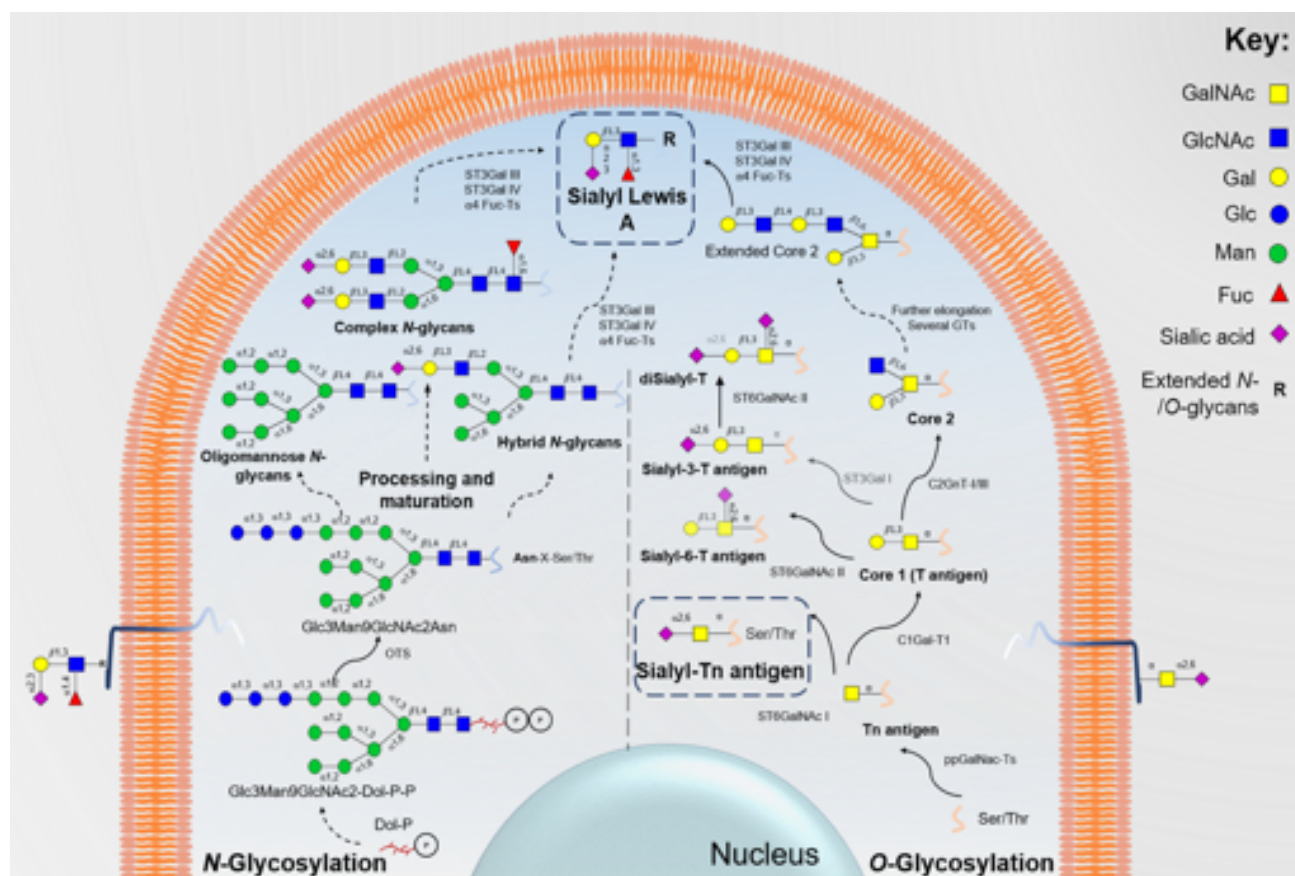


Figure 2. Protein-associated glycan biosynthetic pathways. *N*-glycan biosynthesis begins at the endoplasmic reticulum (ER) membrane with the Glc3Man9GlcNAc2-P-P-Dol precursor transfer to Asn in Asn-X-Ser/Thr sequons of proteins by oligosaccharyltransferase (OST). Of note, “X” represents any amino acid except Pro. Subsequent *N*-glycan processing reactions take place in the ER from where glycoproteins exit to the Golgi apparatus carrying *N*-glycans with either eight- or nine-Man residues, termed oligomannose *N*-glycans. Further, biosynthesis of hybrid and complex *N*-glycans occurs in the medial-Golgi. Subsequent sugar additions diversify the repertoire of hybrid and complex *N*-glycans by elongation of branching GlcNAc residues, capping of elongated branches and *N*-glycan core sugar addition. Common terminal structures include sialyl lewis antigens, such as sialyl lewis A (SLe^A) through the concerted action of beta-galactoside- α -2,3-sialyltransferase-III and IV (ST3Gal III and IV) as well as α -4-fucosyltransferases (α 4-FucT).

Mucin type *O*-glycan biosynthesis is initiated in the late ER or in Golgi compartments by the Polypeptide GalNAcT- mediated transfer of *N*-acetylgalactosamine (GalNAc) to Ser or Thr residues of

proteins in a tissue- and cell-type-specific manner. This first biosynthetic steps yield the Tn antigen (GalNAc α 1-Ser/Thr), the simplest form of mucin-type *O*-glycosylation. Tn antigen can be sialylated into the sialyl Tn (STn) antigen by *N*-acetylgalactosaminide α -2,6-sialyltransferase I (ST6GalNAc I), abrogating further chain extension, or it can be extended into the core 1 antigen (T antigen) by *N*-acetylgalactosamine 3- β -galactosyltransferase 1 (C1Gal-T1). In turn, the core 1 structure can be capped with sialic acid residues through the action of *N*-acetylgalactosaminide α -2,6-sialyltransferase 2 (ST6GalNAc II) or ST3Gal I, giving rise to sialyl T and disialyl T antigens, again preventing further extension. On the other hand, T antigen can be extended into the core 2 glycan by core 2 β -1,6-*N*-acetylglucosaminyltransferase-I or III (C2GnT-I, III). Extended *O*-glycan structures beyond the core 2 antigen can also display terminal structures similar to *N*-glycan such as SLe^A.

This results in loss of normal cell-cell recognition between mucosal epithelial cells and lymphoid cells and gain of E-selectin binding capacity of cancer cells, ultimately favouring immune escape and metastasis [67]. Similarly, non-malignant colon cells predominantly yield the sialyl-6-sulfo lewis X, while cancer cells often overexpress SLe^x [68]. Malignant transformation drives this shift by downregulating sulfate transporters [69], sulfotransferases and PAPS synthases [70]. Overall, malignant transformation promotes the expression of sialylated lewis antigens, which constitute the minimum requirement for selectin binding to their counter-receptors, establishing the basis for hematogenous metastasis [64].

In summary, there is a substantial amount of evidences supporting a functional role for STn and sialylated Lewis antigens, with emphasis in SLe^A, in cancer development. Such rationale associated with a restricted expression pattern in healthy tissues, makes of these glycans a good starting point for developing targeted therapeutics. However, a more precise knowledge about the nature of the glycoproteins carrying these PTM signatures is required envisaging the molecular context for personalized intervention.

Biological significance of STn antigen expression in cancer

As previously described, modulation of glycosylation pathways towards aberrant glycan expression in cancer cells can lead to the neo- and/or overexpression of STn antigen [33, 34, 47, 71, 72]. STn expression has been described in many types of epithelial cancer including OC [73, 74], GC [75] and CRC

[76], and a relevant effort to disclose its functional role has been employed in the last few years. Notably, most studies relayed on glycoengineered cell lines to demonstrate STn functional impact in cancer, since the antigen is rarely expressed at high levels in cell culture settings [34, 53, 77]. Such observations emphasise the intricate relationship between STn expression and the tumour microenvironment [47], which remains to be fully disclosed. In fact, in bladder cancer, it has been suggested that oxygen levels may promote STn biosynthesis by suppressing the expression of glycosyltransferases involved in *O*-glycans elongation [47]; however, the microenvironmental factors driving STn expression remain mostly unknown and should be comprehensively addressed in future studies. To this date there are no functional studies about the role of STn in OC and most functional findings in CRC are inferred from *in situ* tissue studies. Nevertheless, GC cells glycoengineered to overexpress *ST6GALNAC1* and, consequently the STn antigen, displayed enhanced metastatic ability in nude mice than the corresponding negative controls [78]. Moreover, MUC1 and CD44 were found to be major STn carriers, suggesting that STn might negatively impact on GC cell adhesion towards more metastatic phenotypes [78] (**Figure 3A**). Furthermore, GC cells induced to overexpress *ST6GALNAC1* and *ST6GALNAC2* displayed lower proliferation rates than control cells, mostly due to an increase in the percentage of apoptotic cells and not to a G1 cell cycle delay [77]. STn expressing cells also showed markedly reduced homotypic cell-cell aggregation but increased adhesion to ECM components as collagen and fibronectin [77] (**Figure 3A**). In addition, transfected cells displayed increased invasion in a STn dependent manner and MUC1 was consistently found as a major carrier [77]. Other studies have disclosed a novel STn-dependent mechanism for chemotherapeutic resistance in GC cells [79]. Namely, STn altered galectin-3 subcellular localization by decreasing its interaction with cell surface glycan receptors and increasing its intracellular accumulation. In this process, STn decreased chemotherapy sensitivity of cancer cells, highlighting the importance of developing novel strategies targeting galectin-3 and/or ST6GalNAc-I in GC [79] (**Figure 3A**). In addition, studies involving glycoengineered *COSMC* knock-outs demonstrated that *O*-glycan shortening was accompanied by the acquisition of mesenchymal-like traits, while promoting transcriptomic remodelling towards

overexpression of *SRPX2* and *RUNX1* genes (both commonly upregulated in GC tissues) [34]. STn overexpression also lead to the activation of ErbB tyrosine kinase receptors ErbB2 and EGFR in these models, implicating STn in the constitutive activation of oncogenic signalling in GC [34] (**Figure 3A**). *COSMC* knock-out mouse xenografts demonstrated decreased survival compared to wild type xenografts, vowing for the more aggressive nature of short-chain *O*-glycan overexpressing GC cells [34]. These findings highlight the role of STn in the acquisition of malignant features related to increased migration, invasion and chemoresistance of GC.

In addition, overexpression of *ST6GALNAC1* enhanced STn expression in CRC stem cells (CSC) [80]. Particularly, STn was carried by the CSC marker CD44, and increased the sphere-forming ability and resistance to chemotherapeutic agents of CRC-CSC [80]. Furthermore, *ST6GANAC1*-overexpressing cells constitutively activated the Akt pathway, which was blocked by *LGALS3* (Galectin-3) gene knockdown. These findings suggest that *ST6GALNAC1* has a role in the maintenance of CRC-CSCs by activating the Akt pathway in cooperation with galectin-3 and that *ST6GalNAC1* or STn antigen might be reasonable molecular targets for CSC-targeted therapy [80]. Similarly, other studies reported CD44 stem cell markers as a major STn carrier in colorectal cancer [81], suggesting that CD44 glycosylation may be implicated in cellular adhesion alterations. The observations made for GC and CRC agree with previous reports from different types of tumours (breast, ovarian, prostate, bladder) where STn was also implicated in key oncogenic features favouring invasion and metastasis development, supporting the pancarcinoma and aggressive nature of this PTM [23, 59, 82].

Biological significance of SLe^A antigen expression in cancer

As previously mentioned, the overexpression of both SLe^A and SLe^x is associated with increased tumour cell malignancy. Particularly, these glycoepitopes are overexpressed in several types of cancer cells, including oesophageal [83], gastric [84] and colorectal cells [85, 86], where its functional role is being progressively disclosed. Furthermore, SLe^{A/x} are E-selectin ligands in vascular endothelial cells, facilitating tumour cell intravasation into primary tumour blood vessels and circulating tumour cells arrest in distant locations during

hematogenous metastasis [87-89] (**Figure 3B**). Also, interaction of these ligands in colon cancer cells with P-selectin on platelets is thought to facilitate hematogenous dissemination by protecting tumour cells from sheer stress in circulation and immune recognition by natural killer (NK) cells [90] (**Figure 3B**). Also, it potentiates tumor cell adhesive interactions with extracellular matrix proteins of vessel walls, mediating extravasation [91] (**Figure 3B**). Moreover, through this interaction, activated platelets may release platelet-derived growth factors capable of stimulating the growth of highly metastatic colon cancer cells [92]. In addition, colon cancer cells undergoing epithelial-to-mesenchymal transition (EMT) upregulate.

SLe^{A/x} levels and E-selectin binding capacity, while enhancing VEGF production [93]. These findings suggest a pro-angiogenic feedback resulting from cancer cells SLe^{A/x} and endothelial cells E-selectin interactions. The molecular mechanism underlying the EMT-associated SLe^{A/x} expression in colon cancer cells included the transcriptomic regulation of glycogenes in a c-Myc and CDX2 dependent manner. Namely, the EMT-derived enhanced transcriptional activity of c-Myc resulted in upregulation of *ST3GAL1/3/4* and *FUT3*, while down-regulation of *CDX2* suppressed *FUT2* transcription, ultimately leading to SLe^{A/x} overexpression [93].

In GC cells, the mitogenic ErbB2 tyrosine kinase receptor (RTK) was found to be a major carrier of SLe^A, mostly in a *FUT3* dependent manner. Moreover, these cells displayed a hyperactivation of ErbB2 and other members of the ErbB family [94]. However, whether ErbB2-specific glycosylation plays an active part on dimer formation remains to be fully disclosed, although similar associations have reported for other ErbB receptors [95-98] (**Figure 3B**). Notwithstanding, cellular deglycosylation and CA19.9 antibody-mediated blocking of SLe^A drastically altered ErbB2 expression and activation, suggesting an important role for SLe^A in RTK function in GC cells [94]. On another note, SLe^{A/x} are specific ligands of sialic acid binding adhesin (SabA) that promotes *H. pylori* adhesion to the inflamed gastric mucosa [41] (**Figure 3B**). Moreover, SLe^x expression in the gastric epithelium is further induced during persistent *H. pylori* infection, creating a chronic inflammation state capable of establishing precursor neoplastic lesions of the

gastric epithelium [99]. In this context, sialyl lewis antigens actively contribute to GC associated inflammation.

In summary, sialylated lewis antigens, with emphasis on SLe^A, actively contribute to pre-malignant gastric lesions formation, constitutive activation of oncogenic pathways, hematogenous metastasis and immune scape. These findings support the malignant nature of these cancer specific PTM and highlight the need for novel therapeutic approaches targeting these epitopes.

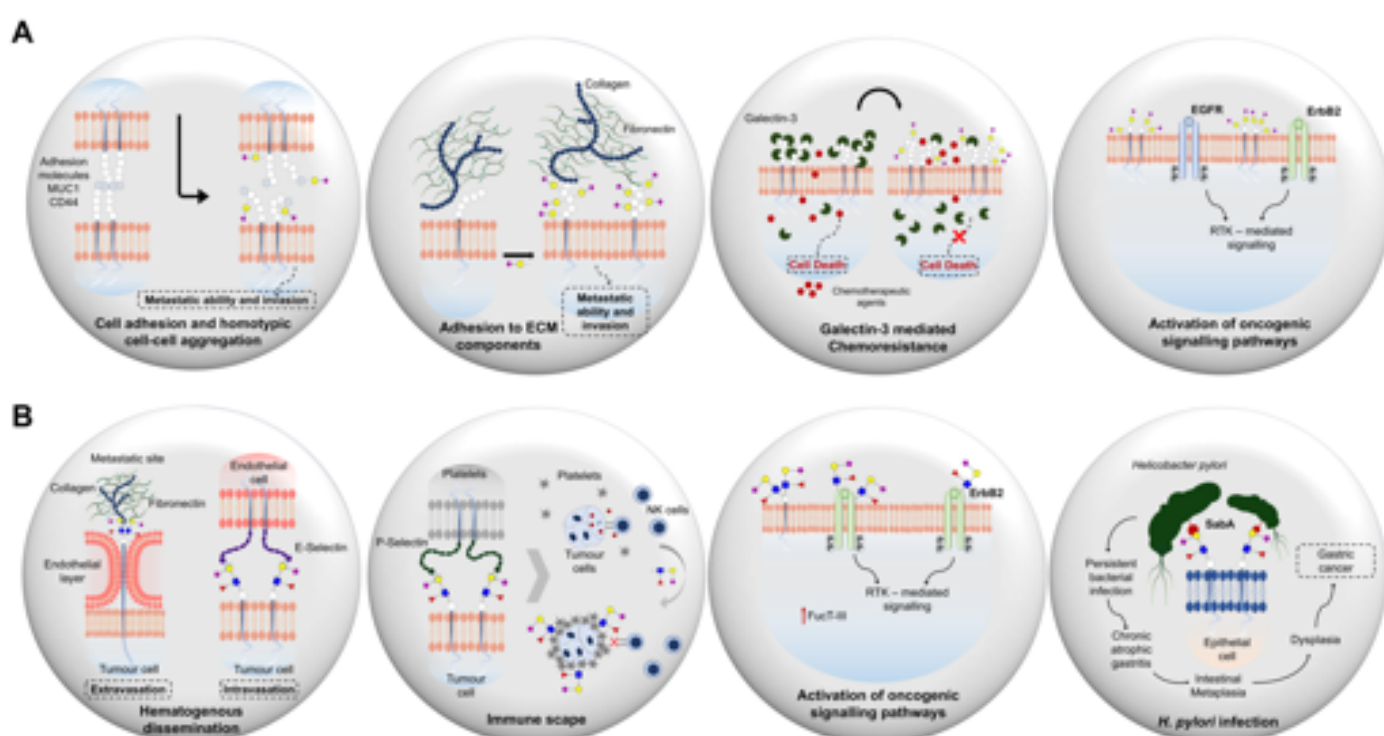


Figure 3. Functional impact of STn (A) and SLe^A (B) expression in gastrointestinal tumours. (A) Adhesion molecules as MUC1 and CD44 are major STn carriers, which negatively impacts cell adhesion towards more metastatic phenotypes. Moreover, STn expressing cells show markedly reduced homotypic cell-cell aggregation, while increasing adhesion to ECM components as collagen and fibronectin. Also, STn alters galectin-3 subcellular localization by decreasing its interaction with cell surface glycan receptors and increasing its intracellular accumulation. In this process, STn decreases chemotherapy sensitivity of cancer cells. STn overexpression also leads to the activation of ErbB tyrosine kinase receptors ErbB2 and EGFR, promoting the constitutive activation of oncogenic signalling. **(B)** SLe^A is the minimum requirement for E-selectin activation in endothelial cells, facilitating tumour cell intravasation into primary tumour blood vessels and circulating tumour cells arrest in distant locations. Also, it potentiates tumor cell adhesive interactions with ECM proteins of vessel walls, mediating extravasation during hematogenous metastasis. SLe^A on tumour cells also interacts with P-selectin on platelets to form cell clusters capable of protecting tumour cells from

sheer stress in circulation and immune recognition by natural killer (NK) cells, facilitating hematogenous dissemination. Moreover, ErbB2 is a major carrier of SLe^A in gastric cancer cells overexpressing galactoside 3(4)-L-fucosyltransferase (FUT3) and displaying receptor tyrosine kinase receptor hyperactivation towards oncogenic signalling. SLe^A is also a specific ligand of sialic acid binding adhesin (SabA) that promotes *H. pylori* adhesion to the inflamed gastric mucosa, creating a chronic inflammation state capable of establishing precursor neoplastic lesions of the gastric epithelium.

Clinical relevance of STn and SLe^A in cancer

Classical serological tests and non-invasive biopsies

Both STn and SLe^A antigens are often present in proteins that are secreted or shed from tumours into circulation, making them accessible for serological detection through the CA72-4 (for the STn antigen) and CA19-9 (for the SLe^A antigen) assays. These tests have been long introduced in clinical practice and frequently support the assessment of gastroesophageal and colorectal cancer patients' status (**Figure 4A**). The CA72-4 test detects a high molecular weight tumour-associated glycoprotein 72 (TAG-72) mucin carrying the STn antigen [100], whereas the CA19-9 in the sera of patients occurs mainly mucins carrying SLe^A [101]. However, in gastrointestinal tumour tissues, the CA19-9 test may detect monosialogangliosides containing SLe^A [101], demonstrating that these antigens may cross-react with SLe^A in a wide variety of glycoconjugates.

Recent meta-analyses clearly associate CA72-4 and CA19-9 serum elevations in GC patients with higher stage and grade, lymph-node metastasis, vessel-invasion and the presence of distant metastasis [102]. Despite showing potential for accessing recurrence, distant metastasis, predicting patient survival, poor response to chemotherapy and monitoring after surgery [103] these markers are not considered suitable for early GC detection. In fact, both show increase in non-malignant conditions that decisively bias results. Namely, the CA72-4 antigen has been found elevated in the serum of approximately 7% of healthy individuals as well as patients with gastric ulcer, gastric polyps, atrophic gastritis and *Helicobacter pylori* infection in a large study involving 7757 adults [104]. These results indicate that routine screening of CA72-4 levels in asymptomatic patients may be ineffective

due to low sensitivity and low positive predictive value [104]. In addition, the SLe^a antigen detected by the CA19-9 test is synthesized and secreted in low amounts by normal human pancreatic and biliary ductal cells and by the gastric, colon, endometrial and salivary epithelia [105]. It is also overexpressed in several benign gastrointestinal disorders, increasing the number of false positives [106]. False negatives may also occur for individuals with Lewis (a-b-) genotype [105], which impacts negatively on the capacity to accurately discriminate cancer patients. Attempts to combine several classical serological biomarkers such as CEA, CA19-9 and CA72-4 in GC also did not provided satisfactory results for early diagnosis [104].

A large metanalysis involving 6434 CRC patients showed that high serum CA19-9 levels before treatment were significantly associated with decreased overall-, disease-, progression- and recurrence-free survival, irrespectively of the geographical region, analysis type, sample size, and treatment methods [107]. Several studies also suggested similar associations for CA72-4, but the statistical power of these observations is still limited by the low number of studies [108]. Nevertheless, the sensitivity of both biomarkers is also considered low to support its addition to current standard surveillance strategies for CRC [109]. Contrasting with GC and CRC, the relevance of the CA72-4 and CA19-9 tests in OC is still poorly understood; nevertheless, the few existing studies also suggested associations with more aggressive disease status [110, 111]. Moreover, high CA72-4 levels may be helpful to predict lymph node invasion in resectable esophagogastric junction adenocarcinomas [105]. In summary, current data supports that CA72-4 and CA19-9 serum elevation is a molecular feature shared by subgroups of OC, GC and CRC patients presenting worst prognosis. Such observations denote a common background shared by tumours of distinct molecular and pathological natures. Nevertheless, there is still little information on the nature and origin of the proteins in circulation carrying these PTM, which will be critical to fully disclose and potentiate their clinical meaning.

Recently there has been a growing interest in exploiting circulating tumours cells (CTC) for prognostication, detection of micrometastasis and as a mean to obtain more accurate insights on the molecular nature of the metastasis, as

emphasized by several recent reviews [112, 113] (**Figure 4A**). Moreover, these cells have been observed in the blood of cancer patients with no evident radiological signs of metastasis [114], reinforcing their importance for early and potentially life-saving interventions. On a more comprehensive scale, CTC analysis may be used to complement circulating nucleic acid and exosome analysis [115, 116]. Classically, CTC isolated from patients' blood by different means (flow cytometry; lab-on-a-chips) have been identified based on the expression of epithelial markers EpCAM and pan-cytokeratins and the absence of CD45 hematopoietic marker [112, 117]. Recently, we have demonstrated that the majority of CTC of different origins, including CRC express the STn antigen at the cell-surface and that targeting this glycan could greatly expand the number of isolated CTC, thus increasing the sensitivity of current CTC detection methods [118] (**Figure 4A**). Moreover, STn-CTC were identified in patients without the presence of metastasis that subsequently experienced disease progression, further reinforcing the sensitivity of the methodology [118]. The presence of STn in CTC also provided the missing link between STn promotion of motility, invasion and immune escape and its presence in distant metastasis of advanced tumours [118]. Taking also into account the functional role of SLe^A in E-selectin mediated metastasis [64], it is likely that this glycan may also be present in CTC, which warrants evaluation. These observations are crucial to improve further on liquid biopsies and should be now extended to larger and well characterized patient cohorts. It also provides the rationale for selectively targeting CTC in clinical settings and developing novel glycan-based therapeutics capable of controlling disseminated disease.

Expression in tumour tissues and metastasis

In clear contrast with serological assays, the evaluation of STn and SLe^A in tumour tissues is not part of the clinical routine. Envisaging the basis for glycoproteomics studies, we have comprehensively interrogated the literature regarding the clinical context for STn and SLe^A expressions in gastroesophageal and colorectal cancer **Table 1**.

Table 1. Correlation between SLe^A and STn tissue expression and clinicopathologic variables of gastroesophageal and colorectal tumours.

Tumour Type	Antigen/Antibody	N	Outcome	Ref
Oesophageal Cancer (SCC)	SLe ^A /NS19-9	54	-	(Oshiba et al., 2000)
	SLe ^A /CA19.9	74	Tumor differentiation (P < 0.05) Poor prognosis (P < 0.05)	(Ikeda et al., 1994)
	SLe ^A /Anti-SLe ^A	125	Hematogenous recurrence (p=0.026) Distant lymph node metastasis	(Makino et al., 2001)
	STn/ B72.3	84	-	(Flucke et al., 2001)
	SLe ^A /CA19.9	115	Stromal type of staining: Depth of invasion Tumour size	(Ikeda et al., 1991)
	SLe ^A /CA19.9	52	Stromal type of staining: Peritoneal dissemination (P < 0.05) Lymphatic invasion (P < 0.05)	(Ikeda et al., 1995)
	STn/ B72.3 and TKH2	52	Stromal type of staining: Peritoneal dissemination in undifferentiated tumours (P < 0.05)	(Ikeda et al., 1995)
	STn/ C 1282	242	Overall survival of stage I patients (P= 0.002) Prognosis (P=0.005)	(Victorzon et al., 1996)
Gastric cancer	STn/ HB-STn1	211	Venous invasion (P < 0.05) Overall survival (P < 0.05) Prognosis (P=0.042)	(Terashima et al., 1998)
	STn/ TKH2	60	Depth of invasion (P< 0.01) Tumour stage (P< 0.01) Lymph node metastasis (P< 0.005) Lymphatic invasion (P< 0.05) 5-year survival of advanced cases (P< 0.01)	(Yamada et al., 1995)
	STn/ TKH2	54	-	(Yamashita et al., 1998)
	SLe ^A /CSLEA1	62	-	(Nakagoe et al., 1991)
	SLe ^A /NS19-9	309	Lymph node metastasis (P < 0.005) Recurrence (P < 0.005) Postoperative survival (P < 0.001)	(Nakayama et al., 1995)
	SLe ^A /CA19.9	52	-	(Lorenzi et al., 1997)
	Unspecified	149	Histological type (P< 0.05) Depth of invasion (P< 0.05) Lymph node metastasis (P< 0.05) Venous invasion (P< 0.05) Astler-Coller stage (P< 0.05) TNM stage (P< 0.05) Recurrence (P< 0.01) 5-year survival (P< 0.01)	(Shimono et al., 1994)

	SLe ^A /NS19-9	52	5-year survival (P< 0.05)	(Akamine et al., 2004)
Tumour Type	Antigen/ Antibody	N	Outcome	Ref
Colorectal cancer	SLe ^A /NS19-9	368	5-year disease-free survival (P=0.012) Recurrence	(Portela et al., 2011)
	STn/ TKH2	52	-	(Akamine et al., 2004)
	STn/ TKH2	142	Depth of invasion	(Yamashita et al., 1998)
	STn/ HB-STn1	152	TNM classification (P=0.025) AJCC classification (P=0.049) in transitional tissue	(Vazquez-Martin et al., 2004)
	STn/ B72.3	234	Histological differentiation (P=0.006) Perineural invasion (P=0.041)	(Xu et al., 2015)
	STn/ C 1282	239	Age (P=0.024)	(Lundin et al., 1999)
	STn/ TKH2	111	-	(Nanashima et al., 1997)
- No correlation to clinicopathological variables was found				

To our knowledge only one study has addressed the expression of STn in OC, exploring the B72.3 antibody that detects clusters of STn bound to serine and may also cross react with Tn clusters [119, 120]. The authors reported that strong STn expression (> 35% of the tumour area) was predictive of decreased patient survival [73]. Notably, there are also evidences that the STn antigen may be mildly present in squamous cells of the healthy oesophagus [121] limiting the scope of these preliminary observations. In opposition to OC, the expression of STn in GC is well documented. STn overexpression was correlated with peritoneal dissemination of tumour undifferentiation [122]. Moreover, most studies have strongly correlated STn immunoreactivity with decreased overall survival, with STn being demonstrated as an independent prognostic factor in some study settings [24, 25]. STn staining has also been correlated with venous and lymphatic invasion, depth of invasion, tumour stage, and lymph node metastasis [24, 123]. In addition, strong positive tissue immunoreactivity was associated with an elevation in CA72-4 serological levels [123], suggesting the shed of tumour proteins into circulation. While Victorzon et al. suggested that STn was a valuable tumour marker capable of discriminating early stage patients with poor prognosis, Terashima et al. observed that STn was an independent prognostic factor in advanced GC patients. Notably, Yamashita et al, found no clinicopathological significance for STn expression in GC [124]. Despite these discrepancies, most likely associated with experimental protocols and different cut-off levels of positivity, most studies support that STn antigen is directly linked to GC aggressiveness. Even though significantly overexpressed in GC, the STn antigen has also been observed in pre-malignant gastric lesions [125, 126] and in lower amounts in parietal and goblet cells in the healthy stomach [25, 127, 128].

Studies in CRC reinforce the cancer-associated nature of the STn antigen, which was not detected in tumour-adjacent normal colorectal mucosa, apart from goblet cells [129-133]. Interestingly, in the colon, STn can be detected in colonocytes after de-acetylation by saponification [134, 135]. Moreover, decreased in sialic acids *O*-acetylation on mucins is a sensitive indicator of early malignant transformation [136]. In CRC tumours STn was distributed in the apical cytoplasm or at the cell membranes, with staining becoming more pronounced with increasing extent of

invasion [129]. It was strongly correlated to poor histological differentiation and perineural invasion [131]. STn was also consistently found in transitional tissue, where it was significantly correlated with tumour stage according to the TNM and AJCC classifications [130], and adenomas [131, 133]. Altogether, these findings suggest that STn overexpression occurs earlier in the carcinogenesis process and STn could constitute a useful marker of the preneoplastic stage of colorectal tissue. Moreover, the different staining localization of STn may assist in distinguishing the process of malignant transformation from a diagnostic standpoint. Notably, the presence and extension of STn in CRC did not correlate with high levels of the antigen in the serum given by the CA72-4 test [137]. Collectively the expression of STn in gastroesophageal and colorectal tumours appears to be linked to higher aggressiveness and metastasis development, in agreement with its functional role. Moreover, it holds potential for patient prognostication (**Figure 4B**). Nevertheless, more detailed studies involving larger and well characterized patient cohorts is required, particularly for OC. STn evaluation in the context of response to different types of therapeutic schemes, including targeted therapeutics and emerging immunotherapies with immune-check point inhibitors is also warranted. This together with the inclusion of STn in emerging stratification models will be critical for better patient stratification (**Figure 4B**). On the other hand, the presence of STn antigen in healthy tissues, despite limited, raises concerns related to off-target effects of targeted therapeutics. A comprehensive characterization of the STn-glycoproteome will be critical to identify cancer-specific molecular signatures. Its comparison with the serum glycoproteome will also be decisive for explaining the discrepancies between tumour and serum status at this level and improving the accuracy and scope of the CA72-4 test.

To date, only three studies were identified involving squamous cell carcinomas (SCC), the most common OC histological type, and SLe^A antigen expression. The SLe^A was consensually observed at the cell membrane and in the cytoplasm of malignant cells but not in the surrounding stromal tissue or in normal epithelium, suggesting a cancer specific nature. Oshiba et al. [138] did not find any correlation between SLe^A expression and clinicopathological variables, while Ikeda, et al. [139] and Makino et al. [140] correlated SLe^A with tumor dedifferentiation and

poor prognosis as well as hematogenous and distant lymph node metastasis, respectively. Differences in the clinical history of enrolled patients, including exposure to different therapeutic schemes prior to molecular evaluation and adoption of different antibodies for SLe^A evaluation, may account for the observed differences. In GC, SLe^A was observed at the membrane and cytoplasm of tumour cells but also in the stromal areas between tumour cell clusters. Undifferentiated carcinomas showing stromal staining were characterized by increased depth of invasion and tumour size [141]. Moreover, these gastric tumours were shown to preferably follow the peritoneal dissemination type of metastatic route rather than liver metastasis [122]. Accordingly, the staining pattern of SLe^A helped predicting the prognosis of patients with undifferentiated type advanced gastric carcinoma, while serving as a useful marker for the early detection of GC metastasis. In CRC, SLe^A expression in the primary tumor and the stroma significantly correlated with several clinicopathological variables. Namely, lymph node metastasis, disease recurrence, decreased survival, poor histological differentiation, depth of invasion, venous invasion, advanced Astler- Coller's stage (C2) and advanced post- TNM stage (IV) [142, 143]. However, some studies failed to find clinicopathological significance for SLe^A in CRC [144, 145]. In addition, SLe^A antigen was found highly expressed in goblet cells of non-pathologic colon tissues [146]. Notwithstanding, most studies established SLe^A as a useful marker of tumor aggressiveness and prognosis in patients with advanced CRC. In addition, some studies provided a comprehensive view on SLe^A status by evaluating both tissue and serum levels in CRC patients. Accordingly, SLe^A detection in tumor tissue and serum identified patients at high risk of cancer recurrence and death and could be useful to select patients for adjuvant therapy [137, 147]. However, SLe^A serum levels were low sensitive for cancer patients, partly because the antigenic determinant of CA19-9 serological assay is a sialylated derivative of the Lewis^A blood group antigen and genotypically Lewis^{a-b-} individuals (about 5–10% of the general population) cannot synthesize the SLe^A antigen [148].

In summary, despite some conflicting result, mostly stemming from the use of different antibodies, most studies highlight the cancer-associated nature of STn and SLe^A, relating the presence of this antigens in the tumour and the stroma with more

unfavourable outcomes. Moreover, in most studies, the percentage of positively immunoassayed tumour samples largely exceeded 50% regardless of the tumour model, making this glycoepitopes representative targets. STn expression in tumours was mostly associated with depth of invasion and decreased overall survival, while SLe^A expression was mainly associated with lymph-node metastasis and disease recurrence. These findings are easily correlated with the functional roles of both antigens, in which STn promotes more motile and invasive tumour cell phenotypes and SLe^A is an active mediator of E-selectin mediated metastasis. Notably, most studies were carried out in the Japanese population, with limited representation of other ethnic groups. Moreover, some authors have reported differences in the prognostic value of some molecular markers in colorectal adenocarcinoma in relation with the ethnic origin of the patients [149, 150]. Also, of all mentioned studies, only Nakayama, *et al.* has included advanced CRC regional lymph node metastases in their clinical sampling, bringing a broader notion of glycan staining in disease progression [143]. Future studies should involve the analysis of broader patient cohorts, the standardization of protocols, the inclusion of samples representative from all stages of disease, and the adoption of antibodies with well-defined glycan recognition patterns. Namely, the existence of a wide number of different antibodies with distinct affinities for glycan moieties has constituted a major drawback for biomarker research. Namely, over the years different types of antibodies have been developed for the STn antigen, targeting either clustered or monomeric STn residues, dependent or independent of the linkage to the peptide backbone, many times showing cross-reactivity with other short-chain O-glycans such as the Tn and O-6 sialoepitopes [119, 120, 134, 151, 152]. Moreover, the nature of immunogens has been diverse, including membrane fractions of breast cancer metastasis (clone B72.3), LS-180 colonic cancer cells (clone MLS102) and ovine submaxillary mucins (clones TKH2; HB-STn1; L2A5). For SLe^A this is even more critical, since it is a terminal epitope of different glycans chains of distinct glycoconjugates (O-glycans and N-glycans in glycoproteins; glycolipids), widely diversifying the array of possible immunogens. A comprehensive understanding about the nature of the cancer-associated

glycoproteins carrying these PTM is also required for a rational design of novel antibodies of biomedical and clinical utility.

Glycan-based therapeutic opportunities

Glycan-based immunotherapy

The cell-surface nature of glycans and glycoconjugates (glycoproteins and glycolipids) holds great potential for developing targeted therapeutics, including selective drug delivery, precise inhibition of key oncogenic pathways and immunotherapy [26, 38].

Immunotherapy based on vaccination with short-chain cancer associated glycans is an appealing concept already explored in clinical trials, even though not for gastroesophageal and colorectal cancers (**Figure 4C**). A pentavalent carbohydrate-based vaccine bearing several carbohydrate antigens, including STn, on a single polypeptide backbone, conjugated to keyhole limpet hemocyanin (KLH) and mixed with the QS-21 immunological adjuvant has entered in phase 1 clinical trials for patients with ovarian, fallopian tube and peritoneal cancers [153]. However, the most promising approach continues to be Theratope, an STn-KLH vaccine that reached phase 3 clinical trials for metastatic breast cancer. Despite well tolerated by the patients, vaccination did not translate into an overall benefit in terms of time to progression and overall survival [154, 155]. However, it has been suggested that a prior knowledge of the STn status of the tumour could have been crucial for patients' election and improving the study outcome [156]. The efficacy of these approaches may also be compromised by immunological barriers raised by the STn antigen. In fact, cancer-associated glycans such as the STn antigen present variable immunogenicity depending on the distribution and nature of the glycopeptide chain [157]. Moreover, STn may directly induce immune tolerance, including limited dendritic cell differentiation and induction of T-cell-mediated immunity, which are crucial for efficient cancer therapy [158, 159]. As an example, densely glycosylated MUC1 sialoglycopeptides, frequently explored in the context of vaccine development, cannot be processed by antigen-presenting cells [160] impairing antigen presentation and consequent T cell activation. Nevertheless,

these studies provide important lessons for choosing more adequate glycoepitopes, setting again the emphasis on glycoproteomics. There have also been attempts to overcome glycan-induced immune tolerance by coupling multiple carbohydrate antigens to specific carriers to form either clustered and/or multi-epitope conjugated vaccines [161-164]. Glycans have also been combined with T-cell derived peptides or immunoadjuvant epitopes to produce glycoconjugate vaccines of multicomponent nature [161, 165-167]. Another strategy involves chemical modifications of glycans to improve immunogenicity. As an example, Song *et al.* recently investigated the antitumor ability of KLH-conjugated fluorinated STn analogues against a murine model of colon cancer [168]. According to the authors, vaccine constructs with substitution of two *N*-acetyl by *N*-fluoroacetyl groups in STn significantly prolonged mice survival and reduced tumor burden in the lungs compared with Theratope (STn-KLH). The fluorinated vaccine elicited stronger cytotoxic T cell and Th1 immune responses and tumor-specific anti-STn antibodies capable of inducing complement and antibody-dependent cell-mediated cytotoxicity against human tumor cells, even in the absence of an immune adjuvant [168]. Collectively, these findings suggest that strategic hapten fluorination may significantly improve the efficacy of glycan-based vaccines, even though the exact mechanism governing this immune response remains unknown. In addition, Ragupathi *et al.* described a SLe^A vaccine construct analogous to theratope but containing a pentenyl glycoside of sLe^A hexasaccharide conjugated to KLH [169]. This vaccine adjuvanted by GPI-0100 induced high IgG and IgM titers responsibly by mediating potent complement mediated cytotoxicity against different cancer cells; however, the authors also reported no cross-reactivity against other blood-group related antigens, including SLe^x. In addition, MUC1-derived glycopeptides associated with cancer are also amongst the array of glycoepitopes used in vaccines explored in clinical trials [170-172]; however, with yet limited success. It was proposed that after first vaccination both tumor MUC1 and MHC molecules were reduced, suggesting an upfront response against these cells that if followed by therapy scape [173]. These findings highlight the need to include a diversified array of glycopeptides that mirror tumour diversity. However, surrogate T-cell pre-activation outside the tumor bed, either in culture or by repetitive vaccination, could

overcome tumor escape in MUC1 transgenic mice, offering an alternative approach to improve therapeutic schemes. Furthermore, the effective development of MUC1-based vaccines would be of great interest for patients with gastroesophageal and colorectal tumours that significantly overexpress abnormal glycoforms of this protein [174-176]. Collectively these studies demonstrate the feasibility of glycan-based anti-cancer vaccines but also highlight the importance of more accurate epitope choice and the improved vehicle design, which requires a more profound knowledge of glycan-immune system interactions. The introduction of distinct T helper cell epitopes, Toll-like receptor agonists and other relevant immunogens in vaccine constructs together with the use of liposomes and nanoparticles as delivery systems may also help paving the way for improved vaccine designs [177, 178].

Another emerging approach relates with exploiting chimeric antigen receptor (CAR) T cells engineered to target glycosylated moieties in cancer cells, promoting selective cell death [179] (**Figure 4C**). Despite boosted by advances in cell engineering, this is an old concept explored for therapeutic proposes in oncoglycobiology. In fact, the first-generation of CAR-T retrovirally transduced to efficiently target the TAG72 glycoprotein in gastrointestinal tumour cell lines dates back to the nineties [180, 181]. Nevertheless, the concept has only been recently translated into a clinical trial for metastatic CRC [182]. However, CAR-T cells were not able to elicit clinical response, potentially due to CAR antigenicity related to the murine origin of the scFv [183], lack of T cell co-stimulatory signalling, vowing for the inclusion of co-stimulatory molecules in CAR design, or the modest affinity of the CC49 anti-STn monoclonal antibody explored by this study [184]. In addition, Loureiro et al. has recently reported that CAR T cells can be efficiently and safely targeted to STn-expressing cells exploiting the recently developed L2A5 monoclonal antibody [185]. These have been effective against breast- and bladder-associated tumor cells both *in vitro* and *in vivo*, but were not yet tested in digestive tract tumours [60]. Loureiro et al. also reports some degree of cross-reactivity between some of the most explored anti-STn antibodies and immune cells. According to this study, L2A5, B72.3 and 3F1 (also referred to as HB-STn-1) showed no affinity for NK cells; however, B72.3 and 3F1 reacted with CD4+ and CD8+ T cells but not B cells. The L2A5 antibody recognized B cells and showed weak binding

to a subpopulation of CD4^{low} T cells [60]. These critical observations demonstrate that CAR-T cells based on these antibodies carry the potential risk of fratricidal activities against T- and/or B lymphocytes. There are also concerns that STn-targeted CAR-T may significantly react against inflamed tissues, known to upregulate this glycan (Ishino, Kawahito et al., 2010, Itzkowitz, Young et al., 1996). Collectively, these observations suggest that, even though vaccination and consequent induction of circulating STn antibodies have been proven safe (O'Boyle, Zamore et al., 1992, Sandmaier, Oparin et al., 1999), more potent CAR-T therapy might encompass significant and potentially limiting off-target effects. Consequently, the future of glyco-targeted CAR-T remains dependent on the identification of targetable glyconeantigens.

Antibody-based therapeutics

Several monoclonal antibodies exist to target both the STn and SLe^a antigens [186, 187], which may be used to induce antibody-dependent cellular cytotoxicity (ADCC) [188-190], a mechanism by which many clinically available therapeutic antibodies promote anti-tumour effects [191, 192], or block relevant oncogenic receptors [94] (**Figure 4C**). These antibodies have been key tools for biomedical research but have shown limitations for theragnostics (cancer detection and therapy), including guiding drugs, CAR-Ts and immunotherapeutic agents to tumour sites with minimal off-target effects. Again, the refinement of the glycoimmunogens poses as a critical milestone towards this end. In addition, the identification of glycoepitopes involved in interactions with the immune system may lead to the development of novel antibodies for immune-check point inhibition; however, this remains a rather unexplored field of research. As such, antibody-targeted therapies for glycoconjugates remains intimately dependent on the development of bispecific antibodies targeting glycodomains in functionally relevant proteins.

Glycosylation inhibitors and mimetics

Another appealing concept is to selectively inhibit glycan-receptor interactions or abrogate glycan biosynthesis pathways (**Figure 4C**). As an example, the GMI-

1271 E-selectin antagonist may be used to inhibit SLe^{A/X}-expressing cancer cells adhesion to endothelial cells, consequently preventing metastasis development [193]. This approach is currently being exploited for acute myeloid leukemia in phase III clinical trials (NCT03616470) [194]; however, given marked similarities in metastasis routes, this approach may be translatable for solid tumours. Other phase 1/2 clinical trial (NCT02952989) was testing 2-fluorofucose (2-FF) [195]; a known fucosylation inhibitor [196], together with pembrolizumab (anti-PD-1) in a wide array of tumours, including gastroesophageal junction adenocarcinomas, colorectal neoplasms and gastric adenocarcinoma. However, the trial was prematurely terminated due to overall benefit/risk profile. There are also promising attempts to use sialic acid mimetics to abrogate sialylation, interfering with sialylated-cell receptors that mediate immune-suppressive environments, ultimately enhancing cytotoxic T cell tumour immunity [197]. These studies support the current relevance of glycomimetics in cancer treatment; however, concerns exist that their untargeted nature may significantly interfere with glycan-mediated cellular homeostasis, which warrants more in-depth evaluation. Nevertheless, conjugation of the molecules with monoclonal antibodies may provide the necessary means to target them to cancer cells.

Glycan-targeted nanovehicles

Targeting nanovehicles carrying therapeutic molecules (chemotherapy, siRNA, small molecules of different natures, etc) to cancer cells is also an appealing therapeutic strategy (**Figure 4C**); however, glycosylation has been less explored for this purpose. Nevertheless, we have recently developed a biocompatible targeted nanoparticle for selective delivery of chemotherapy (5-FU and paclitaxel) to SLe^A-expressing GC cells, with minimal off-target affinity for healthy tissues. We anticipate that the preferential accumulation of nanomolecular constructs in tumour sites, due to ineffective vasculature and poor lymphatic drainage, associated to its targeted effect may significantly improve the controlled release of different agents in tumour sites with minimal toxicity for other organs [198]. The refinement of these solutions *in vivo* and the adoption of bispecific monoclonal antibodies for targeting may pave the way to reduce the adverse systemic effects

associated with chemotherapy, while enabling the administration of biologically effective drug dosages. These approaches may be particularly critical for addressing elderly populations that generally do not tolerate conventional chemotherapy.

From another perspective, a prior knowledge about the structural alterations and functional implications of glycosylation in known cancer targets may help reshaping current antibody-based targeted therapeutics. In fact, many cell surface receptors such as HER2, VEGFR2 and EGFR explored in the clinics are heavily *O*- and/or *N*-glycosylated and may be prone to change their glycode with the progression of disease [29]. Ultimately these glycoproteins may present STn or SLe^A antigens [29, 94], which warrants careful investigation. In addition, glycans influence PD-1/PD-L1 interactions [199]. Namely, PD-L1 glycosylation stabilizes and modulates its binding to PD-1 [200] and efforts are ongoing to exploit the glycoepitopes mediating these processes for patient stratification [201]. A comprehensive glycomapping of these proteins may be helpful for early identification of treatment-responders and designing more appropriate antibodies.

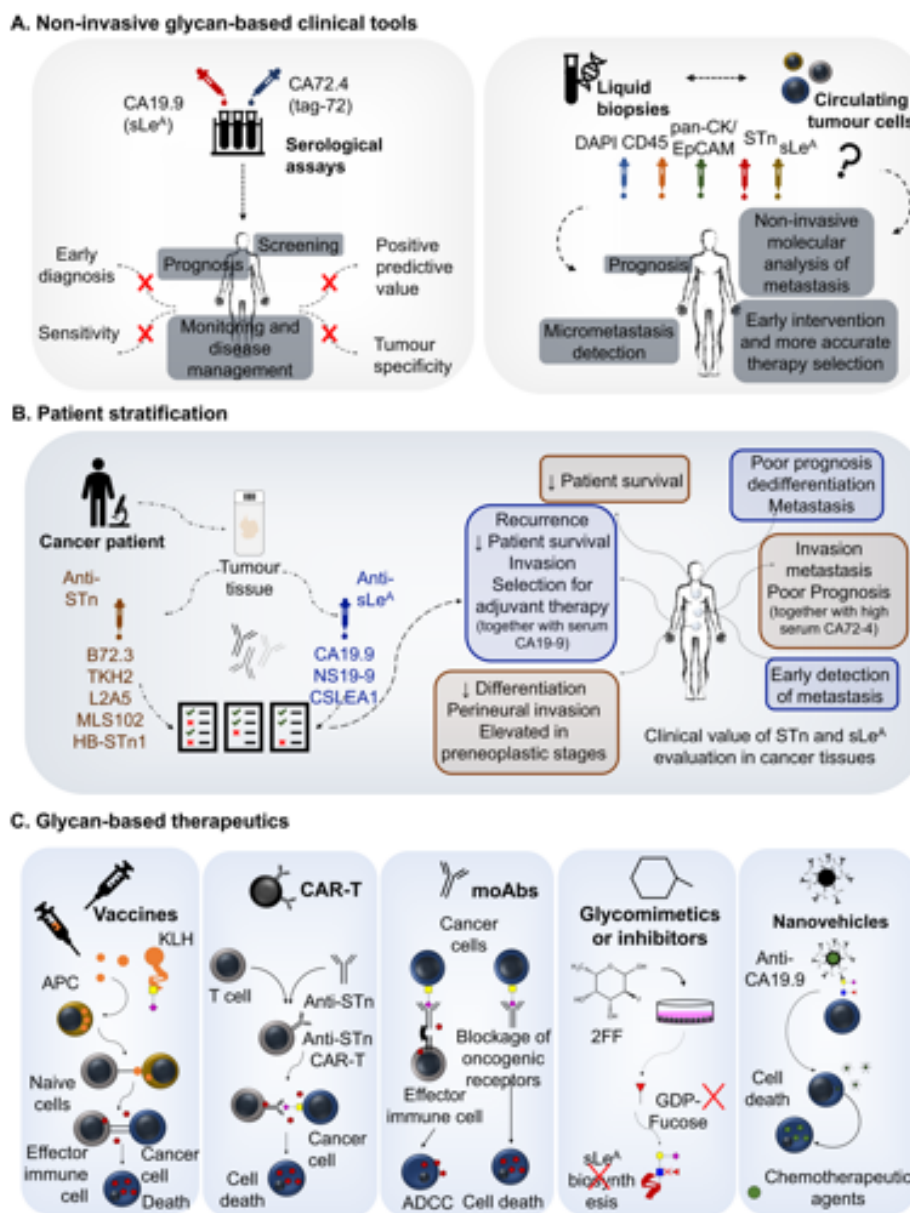


Figure 4. STn and SLe^A clinical applications (non-invasive serological methods; molecular-assisted decisions using cancer tissues; glycan-based therapeutics) in gastroesophageal and colorectal cancers. **A)** Non-invasive serological methods. The STn antigen is detected in the serum using the CA72-4 test; whereas the SLe^A antigen can be detected by the CA19-9 test. These antigens are used for prognostication, cancer screening and response to therapy monitoring. However, both lack the sensitivity and specificity for early diagnosis; **B)** **Molecular-assisted oncology using cancer tissues.** The right side of B panel highlights some of the most explored monoclonal antibodies detecting STn (orange) and SLe^A (blue), while the left side summarizes known clinical associations. **C)** **Glycan-based therapeutics.** This includes glycan-based vaccines such as Theratope, exploiting the STn-antigen linked to a KLH protein carrier to elicited immune responses against STn-expressing cancer cells through antibody mediated killing and cytotoxic T cells effects. Other emerging

immunotherapy is based on CAR-T cells engineered to target cancer cells expressing abnormal glycosylation. There are also several monoclonal antibodies capable of targeting abnormally glycosylated cells promoting antibody-dependent cellular cytotoxicity (ADCC) or blocking relevant oncogenic receptors at the cell-surface. Glycomimetics able to interfere with glycan biosynthesis or blocking glycan-receptor interactions relevant in cancer have also been developed. Finally, antibodies targeting glycans have been used to guide nanoparticles to tumour sites; thereby improving therapeutic outcomes.

Glycoproteomics towards Precision Oncology

The characterization of protein glycosylation is crucial for better understanding the complexity of biological systems and of the utmost importance for next-generation cancer biomarker discovery. In fact, several reports demonstrate that cancer cells present unique glycopeptide repertoires that may significantly improve the clinical value of classical biomarkers. Typical examples include targeting alterations in *N*-glycan antennas and sialylation in prostate specific antigen (PSA) to improve PSA test limited sensitivity for non-invasive detection of prostate cancer [202] or exploring the glycosylation state of MUC16 (CA125) for ovarian cancer [203] (**Figure 5**). However, neither the genome, transcriptome or the proteome can individually or in combination predict the structural nature, distribution and dynamics of glycan chains in proteins. Glycoproteomics attempts to bridge this gap by addressing the glycome, *i.e.* the repertoire of glycans in each biological milieu, as it appears in the proteome. This includes defining which glycosites on a glycoprotein are occupied, identifying and, ideally, quantifying the glycan structures in a peptide chain (**Figure 5**).

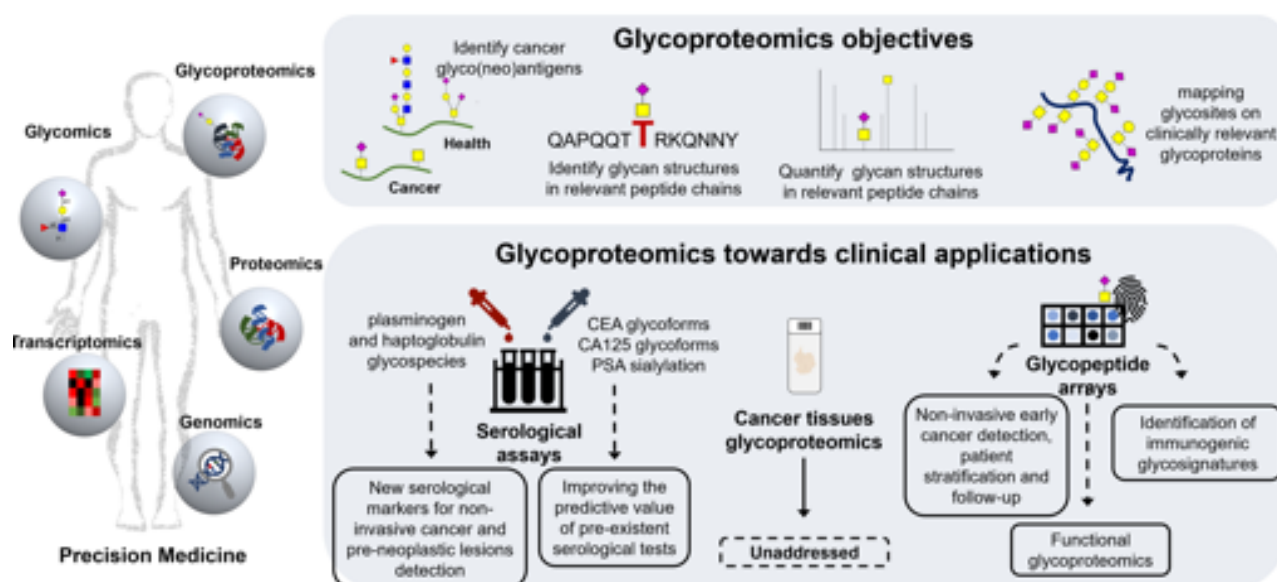


Figure 5. Glycoproteomics objectives and opportunities facing clinical applications. The right top panel summarizes the objectives of glycoproteomics. Glycoproteomics provides an opportunity for identifying cancer unique molecular fingerprints at the cell-surface (glyco”neo”antigens) that are not reflected by healthy cells and non-malignant conditions, paving the way for precise cancer targeting. Alterations in glycan composition, glycosites density and distribution associated to peptide domains in clinically relevant glycoproteins may significantly contribute to this end. The bottom panel highlights the main findings achieved by glycoproteomics in gastroesophageal and colorectal tumours to this date. It mostly includes serological studies, with emphasis on the identification of alterations in the glycosylation of plasminogen and haptoglobin showing potential for non-invasive gastric and esophageal cancer detection and early diagnosis. Targeting specific CEA and CA125 glycoforms may also improve the predictive value of existing clinical tests. In addition, glycopeptide arrays bearing different types of protein glycoforms immobilized in solid supports have shown potential to improve early cancer detection and prognosis based on the identification of autoantibodies. It may also be a relevant tool for identifying potentially immunogenic protein glycoforms as well as a decisive device for functional assays. Contrastingly, cancer tissues glycoproteomics is yet to be initiated, which will be critical foreseeing true clinical applications.

Such goal requires an adaptation of conventional proteomics protocols to accommodate the structural diversity of glycan chains and glycopeptides, which may be particularly challenging since the same protein may exhibit a myriad of different glycoforms. From an analytical perspective, the presence of glycans may significantly decrease the efficacy of proteolysis steps required for conventional routine protein identification by bottom-up proteomics [204, 205]. Moreover, it may negatively influence glycopeptides ionization with consequent reduction in

sensitivity in most mass spectrometers [206, 207]. As such, addressing the glycoproteome may be at the same time a stimulating and daunting enterprise. Nevertheless, a prior knowledge of the glycome is often required to guide glycoproteomics research, enabling the adoption of adequate glycoprotein enrichment methods and MS ionization strategies [208]. In addition, it generates key structural information that tremendously facilitates the annotation of glycopeptides and glycosites using spectral information from tandem MS experiments [209]. These technologies are now sufficiently mature, especially for glycomics, and backed by important protocol guidelines to support clinical studies [210].

For a long time, altered glycosylation in the gastroesophageal and colorectal tracts was mostly associated to mucins due to their macromolecular nature, high *O*-glycosite density and overexpression in cancer [211]. These events were regarded as responsible for amplifying alterations occurring in glycosylation pathways, including the events leading to STn and SLe^A overexpression. However, recent developments in glycoengineering exploiting zinc finger nuclease targeting of *COSMC*, a C1GalT1 chaperone that controls *O*-glycan elongation, has generated several cell lines expressing the Tn and STn antigens, including GC models [34, 212]. This enabled the identification of several hundreds of proteins and thousands of glycosites, which greatly expanded our understanding of GC cell *O*-glycoproteome [33, 58, 213]. Moreover, it demonstrated that abnormal glycosylation such as the Tn and STn may occur in several hundreds of human proteins, including classical cancer biomarkers such as CD44 [214]. Downstream studies on gastric and colorectal tumours set the rationale supporting the decisive role of these glycans in protein function favouring cancer progression and dissemination (acquisition of mesenchymal traits, activation of oncogenic pathways, higher metastatic potential) [22, 215, 216]. Surprisingly, the glycoproteome of gastroesophageal and colorectal tumours remains unexplored and most of the studies involving these models are of serological nature with the goal of improving non-invasive cancer detection. Namely, Gomes et al. has used a targeted glycoproteomics focusing on short-chain T and STn antigens to study a subset of patients with gastritis, incomplete and complete intestinal metaplasia and

healthy individuals envisaging biomarkers of GC precursor lesions [125]. The STn antigen was found in circulating plasminogen in patients with gastritis and incomplete metaplasia, mimicking the expression of STn in gastric tissues [125]. Notably, plasminogen has been associated with chronic infection of the gastric mucosa by *H. pylori* and plays a role in extracellular matrix remodelling and degradation, suggesting that altered plasminogen glycosylation in the serum may be the reflection of the pre-malignant alterations occurring in the gastric microenvironment [125, 217]. This study also clearly emphasizes that revising classical glycan biomarkers from a glycoproteomics perspective may generate a wide array of molecular signatures holding potential for improving non-invasive monitoring of populations at higher risk of developing GC. In addition, Kim *et al.* demonstrated that, haptoglobulin isolated from the serum of GC patients presented unique glycopeptides fingerprints in comparison to healthy controls, including specific alterations in the fucosylation of *N*-glycan antennas [218]. Notably, many of the observed cancer-associated glycans suggest sialylated Lewis epitopes that may contribute to CA19-9 elevation; nevertheless, this aspect has not been fully clarified. Haptoglobulin is one of the most important and abundant acute-phase proteins in the serum [219] and alterations in its glycosylation patterns have been previously reported for an onset of different types of inflammatory diseases and cancers, including CRC [220]. Explorative OC glycoproteomics also involving serum samples has pointed out in the same direction. According to Mann *et al.*, cancer induces several alterations in the abundance of glycoproteins involved with local and systemic inflammatory responses, such as haptoglobulin, α -1-acid glycoprotein, fetuin B and proteins associated with extracellular matrix remodelling such as collagen subunits, EMILIN-2, amongst others [221, 222]. These studies also reveal glycopeptide signatures presenting changes in *N*-glycosylation related with overfucosylation and sialylation associated with OC and precursor lesions [221, 222]. For CRC we emphasize an explorative study using lectin-enrichment that highlights elevations in sialylation and fucosylation in complement C3, histidine-rich glycoprotein, and kininogen-1 in comparison to adenoma and normal tissues [223]. Finally, there have been attempts to focus on specific circulating cancer-associated glycoproteins such as the carcinoembryonic antigen (CEA), produced in

gastrointestinal tissues during fetal development, lost after birth and recapitulated in cancer [224]. The CEA serological assay has been used in clinical practice to monitor response to therapy and recurrence after surgery in CRC [225-227] and in pre-operative settings for aiding tumour staging, treatment definition and prognosis [228]. Nevertheless, it presents limited sensitivity facing early stage disease detection [229] and is falsely elevated in the first weeks after chemotherapy [230]. Moreover, it is elevated in non-cancer-related conditions limiting its application to population screening [231]. However, glycomics studies demonstrate the existence of tumor-specific CEA glycoforms that may be exploited to improve its predictive potential [232, 233]. Nevertheless, collectively, serological studies conducted to this date demonstrate that the serum glycome and glycoproteome may be primarily shaped by glycoproteins resulting from systemic inflammatory responses and, to less extent, by proteins originating from tumour cells and stroma [234, 235]. This fact may help explaining the lack of sensitivity of most common glycan-based tests and the importance of zooming in on the glycoproteome for abnormally glycosylated proteins or even specific peptide domains associated to the disease status.

Exploiting a different perspective, serological studies have shown the existence of humoral responses against abnormally glycosylated cancer-associated proteins in CRC patients. Pedersen et al. used mucin glycopeptide arrays to demonstrate the existence of autoantibodies against aberrant glycopeptides derived from MUC1 and MUC5 with Tn, STn and core 3 glycans in the serum of cancer patients. The authors describe that the cumulative sensitivity of the array analysis was 79% with a specificity of 92% for CRC detection [157]. Moreover, autoantibodies to truncated Core3-MUC1 could be detected in both CRC and inflammatory bowel disease, suggesting this may constitute early steps of altered glycosylation in colon tissues that are not reflected in the healthy condition. In addition, our group has used patients' autoantibodies to demonstrate the existence of protein signatures carrying the SLe^A antigen in OC that changed according to the severity of the disease; however, the nature of these glycoproteins remains to be elucidated [236]. These preliminary observations strongly suggest that glycosylation may contribute to originate cancer neoantigens that ultimately lead to an immune response;

however effective tumour elimination by the immune system is likely not to occur due to the immunosuppressive cues promoted by short-chain O-glycans. Nevertheless, the notion prevails that humoral response may be used to pinpoint cancer-associated glycan signatures.

In summary, the glycoproteome of gastroesophageal and CRC tissues remains unaddressed. The studies conducted so far are focused on glycoengineered cell lines or patient's serum and are of explorative analytical nature. Moreover, most included a very low number of patients of diversified clinical histories and employed distinct protocols. Nevertheless, there are strong suggestions that cancer induces profound alterations in the serum glycoproteome that are not reflected in healthy and benign conditions. Notably, most of the identified alterations relate with systemic responses and extracellular matrix remodelling accompanying cancer development, rather than specific proteins originated from tumour cells. A refinement of these studies by targeted approaches for cancer-associated glycans may provide the necessary settings for improving further on the potential of glycan-based non-invasive detection of digestive tract tumours. It is also surprising that the significant functional and clinical rationale involving STn and SLe^a antigens has not yet been translated in a targeted interrogation of gastroesophageal and colorectal glycoproteomes, including primary lesions as well as the metastasis. This has significantly delayed a more comprehensive understanding of cancer progression and impacts negatively on the capacity to develop bi-specific targeted interventions, exploiting the cancer-specific nature translated by bridging the glycome and the proteome.

Panomics for Cancer Biomarkers Discovery: Where do glycoproteomics fits facing precision oncology?

Biomarker research has experienced tremendous evolution over the past five years to accommodate the requirements of precision oncology. As a result, classical strategies focusing on a limited number of targets are being progressively replaced by systems biology frameworks propelled by high-throughput omics technologies. The technological readiness of next-generation sequencing platforms (genomics, transcriptomics) associated to consolidated bioinformatics and improved

computational capacity has enabled the screening of large patient cohorts (**Figure 6**). Genomics and transcriptomics data have been made available in either raw (primary databases) and/or curated formats (secondary databases) in web repositories such as Gene Expression Omnibus (GEO) [237], ArrayExpress [238] and Oncomine [239], enabling fast access, data integration and reinterpretation. In addition, databases such as Molecular Signatures Database (MSigDB) [240], Cancer Genome Project [241], among others, have been exclusively created to gather molecular information related with cancer [242]. As a result, transcriptomics studies on gastric and CRC have been recently reinterpreted and served as bases to establish consensus molecular subtypes (CMS) with distinct clinical behaviours [243, 244]. Namely, in CRC, the CMS1 subtype is immunogenic and hypermutated. CMS2 tumors are activated by the WNT- β -catenin pathway and have the highest overall survival. CMS3 feature a metabolic cancer phenotype and CMS4 cancers have the worst survival, while having a strong stromal gene signature [244]. In turn, the Asian Cancer Research Group (ACRG) reported four molecular subtypes of GC with clinical significance based on mRNA expression profiles: microsatellite-stable (MSS)/TP53⁻, MSS/TP53⁺, MSI, and epithelial-to-mesenchymal transition (EMT) subtypes. In this molecular classification, the MSI subtype was consistently associated with favourable prognosis, while EMT GC showed significantly higher recurrence rate, higher probability of peritoneal seeding at recurrence, younger age at presentation, and poorer survival compared to other subtypes [243]. Notwithstanding, the predictive value of these models is being substantially refined through the continuous integration of clinicopathological data.

Despite the promising nature of multiomics approaches, the spotlight has been mostly centred on the genome and transcriptome, which often fail to fully reflect the proteome. A common example relates with reported discrepancies between gene expression and protein abundance at different levels [245]. On the other hand, cancer proteomics is still constrained in its capacity to identify the products of single-nucleotide variants, fusion genes and alternative splicing, amongst other relevant events that alter protein primary sequences. Such limitations are associated with the lack of representation of genomic alterations in the databases conventionally used for protein annotation from tandem (MS/MS)

mass spectrometry experiments. This limits the capacity of identifying cancer-specific signatures at the protein level, including neoantigens that hold great potential for targeted therapeutics. In recent years, oncoproteomics initiatives are attempting to bridge the genome and the proteome using customised protein databases inferred from genomics (**Figure 6**). The conceptual bases of the approach are beyond the scope of this review but can be found comprehensively detailed in recent publications [246, 247]. Notably, GC and CRC oncoproteogenomics has revealed the vulnerabilities and improved on currently established molecular models and allowed to prioritize several targets previously inferred by genomic analysis, including copy-number drivers and mutation-derived neoantigens. Collectively, oncoproteogenomics decisively demonstrated that the combination of different levels of molecular information is of key importance for targeted intervention.

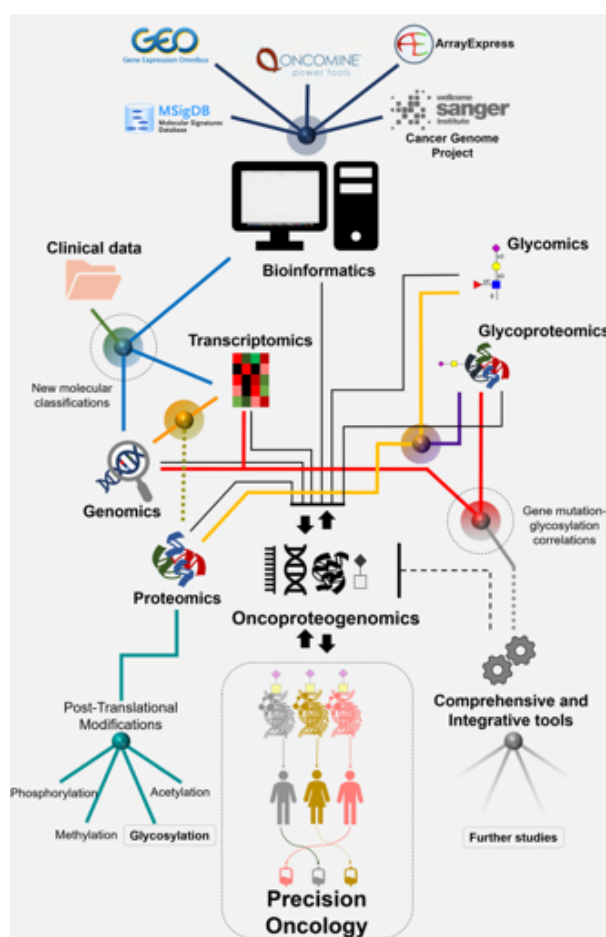


Figure 6. The quest for molecular-assisted precision oncology can be found in the crossroad between interdependent omics (genomics, transcriptomics and proteomics with PTM analysis) backed by comprehensive bioinformatics settings. This panel shows the possible connections

between different omics, attempting to emphasize the importance of bringing together genomics, transcriptomics and proteomics allied to PTM (phosphorylation, methylation, acetylation and glycosylation, amongst others) analysis. It also aims to highlight the decisive role played by bioinformatics and current omics databases, which paved the way for tailored oncoproteogenomics. Namely, the comprehensive integration of genomics intel in customised databases can now greatly expand the coverage of protein annotations envisaging cancer neoantigens. This comprehensive strategy would be of key importance for accurate tumour stratification as well as identification of functional protein nodes and neoantigens for precise cancer detection and therapeutic design.

While oncoproteogenomics sets itself as the next cornerstone in cancer biomarker research, it becomes clear that including post-translational modifications (PTM) in comprehensive panomics models is crucial for better understanding biological milieus. PTM are not direct gene products but they exponentially expand the molecular and functional diversity of the proteome. PTM also facilitate rapid biologic adjustments to altered physiologic demands by enabling rapid incorporation of microenvironmental cues into protein function. For example, phosphorylation promotes the activation (deactivation) of protein functions and acetylation and methylation of histones play a key role in epigenetic regulation of gene transcription, amongst other factors. Showcasing PTM importance, Vasaikar et al. recently identified Rb phosphorylation as a key oncogenic driver of proliferation and decreased apoptosis, providing the rationale for intervention in CRC [248]. Very recently, Dong-Gi Mun et al. provided a proteogenomic analysis of diffuse GC in young populations by integrating mRNA, protein, phosphorylation, and *N*-glycosylation data [249]. Interestingly, clear gene mutation-glycosylation correlations have emerged. Namely, in samples containing significantly mutated *TP53*, *CDH1*, *ARID1A*, and *RHOA*, *N*-glycosylation levels were significantly increased. Moreover, the proteins with such increased *N*-glycosylation were significantly associated with cell migration and immune response-related processes. Similarly, somatic mutations of genes involved in focal and integrin-mediated adhesion, regulation of actin cytoskeleton, and FGF, PI3K-AKT, integrin, and Toll-like receptor signalling, as well as ECM-receptor and integrin interactions, were associated with the *N*-glycosylation of proteins involved in the same processes [249]. These findings suggest that *N*-glycosylation data, like phosphorylation data, provide additional information regarding associations of mutations with cancer-

related cellular processes, reinforcing the key importance of integrating PTM in comprehensive molecular models. Overall, this study demonstrated that the distinction of tumour subtypes would not have been attainable through transcriptomics alone, again reinforcing the need for combining distinct molecular data. It is also pioneer in terms of bringing glycosylation into comprehensive multiomics settings, supporting more in-depth analysis in future studies. Nevertheless, the inclusion of glycoproteomics, with emphasis on STn- and SLe^A targeted studies, in comprehensive panomics models remains an open field of research of unquestionable potential.

Concluding Remarks and Future Perspectives

Gastroesophageal and CRC patients share similar therapeutic challenges that may be significantly attenuated by a better understanding of their molecular nature. Systems biology approaches bringing together different omics with clinical data are considered the next cornerstone for tumour molecular subtyping, enabling the identification of critical protein functional nodes for targeted intervention. Over the past five years, important milestones have been achieved towards this end, including elegant demonstrations that accurate patient stratification can only be achieved through the incorporation of multiple layers of biomolecular information (genome, transcriptome, proteome, PTM). In particular, the development of potentially curable actions is strongly dependent on precise patient stratification and the identification of neoantigens suitable for targeted approaches with minimal off-targeted effects. This is a challenging analytical task to be performed at the protein level, requiring genomics-customized proteomics workflows and the incorporation of PTM, which are critical vectors for defining protein function in response to microenvironmental cues (**Figure 7**). Alterations in protein glycosylation occurring at the cell-surface constitute the next logical step towards this end. Over the past twenty years a significant amount of functional and clinical data has been generated linking STn and SLe^A antigens to cancer aggressiveness in many distinct models, including gastroesophageal and colorectal carcinomas. As a result, serological tests have been created for these antigens and, despite sensitivity and specificity limitations, they still serve us well in clinical practice for prognosis and patient

monitoring. Very recently, the STn antigen was discovered in CTC and preliminary studies suggest it may also be useful for improving the sensitivity of this type of liquid biopsies, while paving the way for targeted interventions. The analysis of solid tumours points in the same direction, strongly reinforcing associations between these glycans and poor outcome. Despite the scarce number of reports involving the oesophagus, STn and SLe^A antigens appear to present a pancarcinoma nature, which suggests common microenvironmental drivers that are yet to be disclosed. In parallel, functional studies involving different types of cell lines demonstrated that the replacement of extended *O*-glycans by short sialylated glycosides such as the STn antigen or changing terminal glycan epitopes by sialylated Lewis determinants decisively dictates cell fate towards metastasis. Namely, this occurs by glycan-promoted enhanced motility, invasion of the extracellular matrix and neighbouring tissues, adhesion to lymphatic and venous endothelia and immune escape. The existing functional and clinical background is now sufficiently solid to support interventional actions towards clinical applications.

The sweet side to cancer-associated glycans sour end resides on the fact that they may dramatically alter the protein landscape originating unique cancer fingerprints at the cell-surface, as elegantly demonstrated by several studies [47, 94, 208, 214]. In fact, STn and SLe^A are not tumour-specific antigens but exhibit a sufficiently restricted expression pattern in healthy tissues to support biomarker potential. Their reflection in the cancer proteome may, however, provide the necessary path towards cancer neoantigens. The challenge is set on the identification of cancer specific STn and SLe^A glycopeptide moieties, thus overcoming limitations associated with the presence of these glycans in low abundance in non-malignant conditions (**Figure 7**). Nevertheless, comprehensive targeted studies on the tumour STn and SLe^A glycoproteomes have not yet started and currently constitute an outstanding challenge to the field that must be addressed soon. Moreover, approaches to the serum glycoproteome have been of explorative nature and did not yet provided clinical translatable glycobiomarkers. Nevertheless, revisiting the circulating glycoproteins carrying these PTM in the context of health and disease will be crucial to better understand tumour development and decisive for improved serological tests. Moreover, the

identification of functional glycoepitopes of clinical relevance will require the incorporation of glycoproteomics with proteogenomics perspectives, has elegantly highlighted for diffuse type GC (**Figure 7**). Other outstanding challenges facing precision medicine include the identification of glycosignatures to predict treatment outcome. In fact, a careful mapping of relevant cancer cell receptors such as ErbB2, PD-L1 or VEGFR2, which are glycoproteins targeted by therapeutic antibodies, may be critical to define resistance to therapeutics by impaired recognition. For instance, according to Duarte et al., ErbB2 targeted by trastuzumab expresses the SLe^A antigen in GC and cellular deglycosylation and antibody blocking of this glycan significantly altered ErbB2 expression and activation *in vitro*, suggesting that a better understanding of this receptor's glycosylation might be crucial for improving targeted intervention [94]. Moreover, it has been demonstrated that altered PD-L1 glycosylation defines its recognition by PD-1 [200] and this knowledge may be of key importance for selecting patient better served by this immunotherapy or improving current targeting. In a broader sense, alterations in the proteins glycode are determinant for defining the cancer immune status and its metastatic potential by enabling immune escape. It is well established that some subsets of glycoproteins carrying the STn and SLe^A may be recognized by siglecs and selectins on antigen presenting cells and lymphocytes [66, 67, 250, 251], eliciting inhibitory signalling cascades. The identification of this glycoproteins may constitute an important milestone towards novel classes of immune check-point inhibitors. A wide array of reports also supports that the STn antigen is expressed by chemoresistant cells presenting stem-cell phenotypes [252] and functional studies have directly implicated STn in this process. Deeper understanding of the chemoresistance-associated subglycoproteome will be crucial for defining patients better served by alternative therapeutics and designing therapeutic alternatives.

In the context of therapeutics, important pre-clinical studies and clinical trials have explored glycans with exciting up-front results. The approaches are diverse, including glycovaccines, targeted nonvehicles for controlled drug delivery, bi-specific antibodies targeting specific glyco-domains in relevant glycoproteins, and CAR-T cells directed against glycoepitopes, with emphasis on the STn antigen (**Figure 7**). Antagonists and inhibitors are also available to selectively inhibit glycan

biosynthesis, and a wide array of strategies interfere with glycan-lectin interactions, which are central axes of many aspects of cancer progression, including cell proliferation, invasion, extravasation and immune evasion. However, the efficacy of targeted therapeutics focusing on cell-surface glycans remains challenged by the lack of target specificity, making true advances in this field dependent on a reinvestigation of the glycoproteome. More effective immunotherapies will also require a deeper understanding about the mechanisms by which glycans mediate immune tolerance, including the specific nature of the glycoproteins and the full repertoire of immune cells lectins involved in these processes.

In summary, classical glycobiomarkers have served us well in serological settings but still hold a tremendous unexplored potential to improve gastroesophageal and CRC patient's management, regarding that outstanding, yet achievable, milestones are accomplished. The current rationale supports the need to engage in glycan-targeted glycoproteomics screening of large and well characterized patient sets from both the molecular and clinicopathological standpoints envisaging cancer glyconeantigens (**Figure 7**). The success of this approach is directly linked to a better understanding of the human STn and SLe^A-glycoproteome in health and disease and its spatio-temporal evolution over the course of disease management and in response to microenvironmental cues. Reinvestigating the human glycoproteome is particularly critical since our current vision of glycosylation is, to some extent, blurred by the information retrieved from immunoassays based on a diversified array of antibodies with distinct affinities for glycan chains. The known implications of both STn and SLe^A in disease progression also vow for an emphasis on the molecular characterization of the metastasis from this angle. Nevertheless, it is critical to progress beyond proof-of-concept approaches regarding the inclusion of glycoproteomics into multi-omics biomarker research. According to recent findings, this step will be crucial for establishing more comprehensive patient stratification models, the identification of potentially targetable glycobiomarkers and the rationale design of targeted therapeutics.

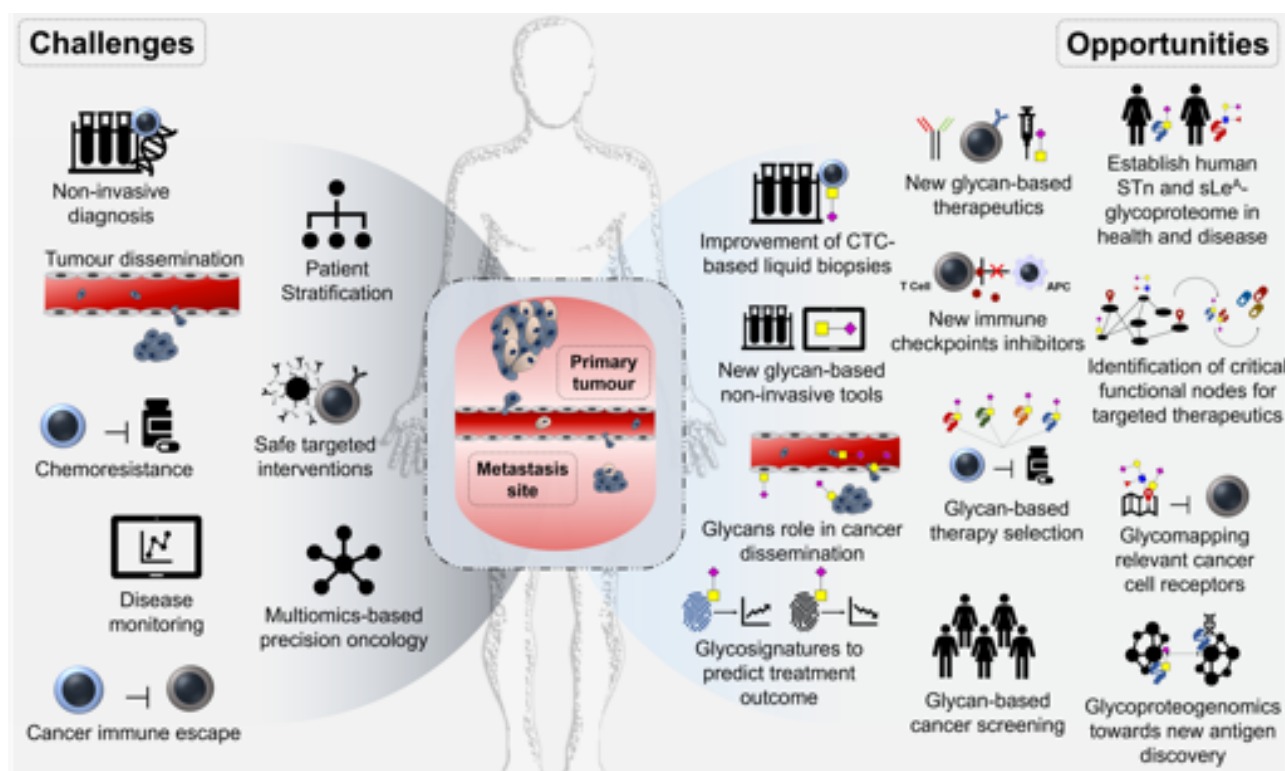


Figure 7. Main cancer challenges tackled by targeted glycoproteomics and glycan-based opportunities facing clinical translation. Glycans, particularly the STn and sLe^a antigens, have been explored in the context of non-invasive cancer diagnosis, patient stratification, response to chemotherapy, disease monitoring, understanding and addressing immune response to cancer cells and design of safer targeted therapeutics. These approaches have been challenged by the lack of tumour specificity of these glycans; nevertheless, these glycobiomarkers specificity is being refined based on the integration of multiomics approaches. Fulfilling this objective will pave the way for improvements in liquid biopsies, targeted therapeutics, non-invasive cancer detection tools, patient stratification and prognostication models, novel targeted therapeutics, new immune check-point inhibitors, cancer glyconeoantigens and ultimately an improved understanding on the role of glycans in health and disease.

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Conflict of Interests

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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New Trends In Guided Nanotherapies For Digestive Cancers: A Systematic Review

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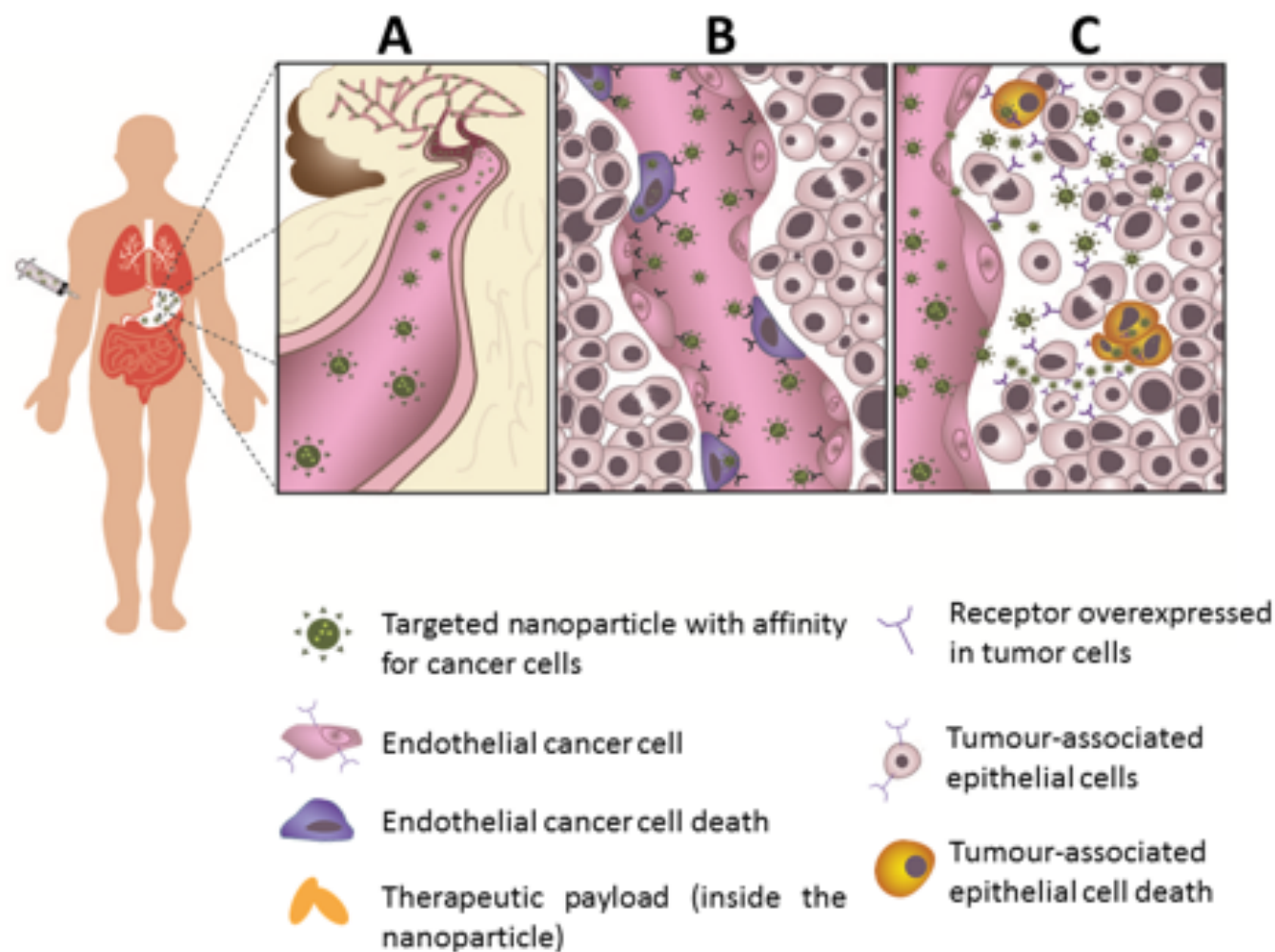
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Running title: Guided nanotherapies for digestive cancers

GRAPHICAL ABSTRACT



ABSTRACT

Digestive tract tumors are among the most common and deadliest malignancies worldwide, mainly due to late diagnosis and lack of efficient therapeutics. Current treatments essentially rely on surgery associated with (neo)adjuvant chemotherapy agents. Despite an upfront response, conventional drugs often fail to eliminate highly aggressive clones endowed with chemoresistant properties, which are responsible for tumor recurrence and disease dissemination. Synthetic drugs also present severe adverse systemic effects, hampering the administration of biologically effective dosages. Nanoencapsulation of chemotherapeutic agents within biocompatible polymeric or lipid matrices holds great potential to improve the pharmacokinetics and efficacy of conventional chemotherapy while reducing systemic toxicity. Tagging nanoparticle surfaces with specific ligands for cancer cells, namely monoclonal antibodies or antibody fragments, has provided means to target more aggressive clones, further improving the selectivity and efficacy of nanodelivery vehicles. In fact, over the past twenty years, significant research has translated into a wide array of guided nanoparticles, providing the molecular background for a new generation of intelligent and more effective anti-cancer agents. Attempting to bring awareness among the medical community to emerging targeted nanopharmaceuticals and foster advances in the field, we have conducted a systematic review about this matter. Emphasis was set on ongoing preclinical and clinical trials for liver, colorectal, gastric and pancreatic cancers. To the best of our knowledge this is the first systematic and integrated overview on this field. Using a specific query, 433 abstracts were gathered and narrowed to 47 manuscripts when matched against inclusion/exclusion criteria. All studies showed that active targeting improves the effectiveness of the nanodrugs alone, while lowering its side effects. The main focus has been on hepatocarcinomas, mainly by exploring glycans as homing molecules. Other ligands such as

peptides/small proteins and antibodies/antibody fragments, with affinity to either tumor vasculature or tumor cells, have also been widely and successfully applied to guide nanodrugs to gastrointestinal carcinomas. Conversely, few solutions have been presented for pancreatic tumors. To this date only three nanocomplexes have progressed beyond pre-clinical stages: i) PK2, a galactosamine-functionalized polymeric-DOX formulation for hepatocarcinomas; ii) MCC-465, an anti-(myosin heavy chain a) immunoliposome for advanced stage metastatic solid tumors; and iii) MBP-426, a transferrin-liposome-oxaliplatin conjugate, also for advanced stage tumors. Still, none has been approved for clinical use. However, based on the high amount of pre-clinical studies showing enthusiastic results, the number of clinical trials is expected to increase in the near future. A more profound understanding about the molecular nature of chemoresistant clones and cancer stem cell biology will also contribute to boost the field of guided nanopharmacology towards more effective solutions.

INTRODUCTION

Gastrointestinal carcinomas are a heterogeneous group of malignancies of the digestive track, which includes namely, esophagus, stomach, liver, pancreas and colorectal tumours, which all together represent one of the major leading causes of death by cancer worldwide. Colorectal, gastric and hepatic cancers not only present the highest incidence but also the most elevated mortality rates [1]. These tumors are often diagnosed in an advanced stage and their rapid metastatic rates constitute a major poor prognosis factor [2]. Furthermore, disease management relies mostly on surgery in association with (neo)adjuvant chemotherapy agents, namely, Anti-metabolites (5-fluorouracil, 5- FU), Topoisomerase inhibitors (Doxorubicin, DOX), Platinum Salts (Cisplatin), Anthracycline drugs (Epirubicin, EPI), Taxanes (Paclitaxel, PTX) and/or radiotherapy [3].

Conventional chemotherapy, while efficient against the tumour bulk, often fails to eliminate subpopulations of highly aggressive cancer cells endowed with chemotherapy resistance, capability of enhance tumour heterogeneity and develop metastasis [4,5]. Drug resistance is considered a multifactorial process highly influenced by inadequate pharmacokinetics of the drugs and abnormal tumour vascularity, resulting in the delivery of suboptimal concentrations of the agents to tumour sites [5,6]. In addition, tumour cells may either present, or develop during the course of treatment, intrinsic molecular mechanisms to overcome the chemotherapeutic challenge [7–9]. Conventional chemotherapeutic agents are highly toxic, therefore limiting the dosage that can be administered to patients, and reducing the efficacy of the treatment [10–12]. Toxicity is a particularly critical matter for the elder population that constitute the majority of the patients diagnosed with gastrointestinal malignancies [13,14], urging the introduction of less toxic and more effective drugs.

Nanoencapsulation of chemotherapeutic agents by biocompatible molecules holds great potential to exceed some of the limitations of

conventional chemotherapeutic agents, namely by enhancing the pharmacokinetics and efficacy of the drugs while reducing systemic toxicity. Moreover, nanoencapsulation may improve the solubility of poorly water-soluble compounds and has the ability to deliver two or more drugs simultaneously upon co-encapsulation in the same nanocarrier [15]. Due to their nanoscale dimensions, nanomedicines preferentially target solid tumours in comparison to healthy tissues by exploiting vasculature imperfections [16]. Solid tumours present tortuous and poorly differentiated blood vessels (100-600nm fenestrations), that in contrast to healthy vasculature (1-2nm fenestrations) allow the extravasation of drugs with sizes up to several hundreds of nanometers [17,18]. Tumors also lack functional lymphatic drainage, making them unable to eliminate extravasated nanomaterials. As a result, long-circulating nanoparticles (NPs) tend to accumulate in tumors over time, a mechanism known as enhanced permeability and retention (EPR) effect (Figure 1A) revised by Maeda et al. [19]).

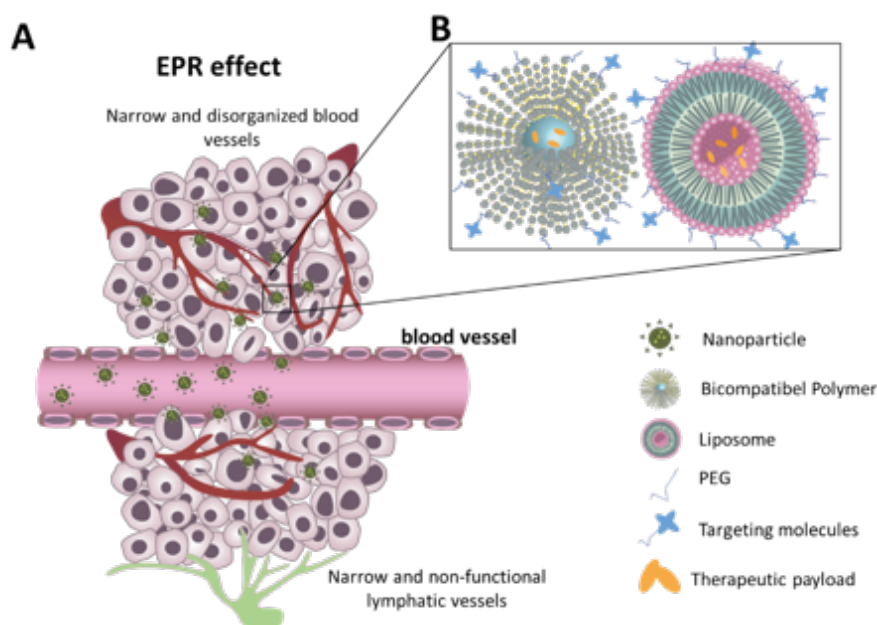


Figure 1. Several chemotherapeutic agents (DOX, 5-FU, cisplatin, EPI, docetaxel, PTX) have been encapsulated in biocompatible polymers (PLGA, PLA, chitosan) or different types of liposomes. The functionalization of the nanoparticles with several ligands (carbohydrates, peptides/proteins, moAbs, and other molecules) with affinity for cell-surface receptors overexpressed at the surface of cancer cells has allowed active targeting of the nanoparticles to cancer cells, thereby improving their therapeutic effect.

There are a variety of NPs systems currently being explored for cancer therapeutics [20], including biodegradable nanocarriers as lipid-based [21], polymeric nanoparticles [22], dendrimers [23], micelles [24], and non degradable nanocarriers, namely carbon nanotubes [25], mesoporous silica [26] and magnetic nanoparticles [27]. Cationic liposomes and biopolymers (Figure 1B) are currently the two major carriers used to complex therapeutic payloads [28,29]. Functionalization of liposomes with polyethylene glycol (PEG) (Figure 1B) or other inert hydrophilic polymers has allowed to reduce their negative-charged surface, and consequently the opsonization and clearance from the blood stream, increasing nanocarrier circulation half-lives [3,30]. Accordingly, Abraxane, a PTX albumin-stabilized nanoparticle formulation, has already been approved for breast, lung and pancreatic cancers and many others face late stage clinical trials [revised by Stirland *et al* [31].

Drug-loaded nanoparticles may accumulate in tumour tissues solely due to the EPR effect (passive nanocarriers) [32,33] or may actively target cancer cells [20,34].

Active targeting is achieved by functionalization of the nanocarriers surface with ligands that specifically recognize and bind to receptors overexpressed on the surface of tumor cells [20,35]. The most common homing molecules include monoclonal antibodies (mAb) or antibody fragments (such as antigen-binding (Fab') or single chain variable fragments (scFv)) [36], peptides and proteins [37], carbohydrates [38] and, more recently, aptamers [39]. This new generation of “intelligent” anti-cancer agents has enhanced target cell recognition, cell uptake and tissue microdistribution in comparison to non-guided nanotherapeutics [6-9,14]. Namely, several reports show that targeted nanoencapsulated drugs preferentially bind to cancer cells within the tumors bulk, whereas non-targeted vehicles accumulated in the tumor stroma, limiting their action [40,41]. The affinity of guided nanodrugs to tumor cells also avoids their translocation back to the circulation, thereby improving their efficacy [35]. Ideal targeted cell receptors should be tumor specific, homogeneously expressed on the tumor cell surface and should not be shed into the blood circulation, which would contribute to reduce the nanodrugs bioavailability [20,42]. Moreover, internalization of targeting conjugates must also occur by receptor-mediated endocytosis after binding to target cells, facilitating drug release inside malignant cells [43–45].

Altogether, guided nanotherapeutics present several advantages in relation to both conventional chemotherapy and non-targeted nanosystems and may constitute a promising tool to improve cancer management. Reflecting this reality, there are approximately 12000 articles deposited on PUBMED regarding the keywords “nanoparticles” and “cancer”, most of them published in the last 5 years, which emphasizes the exponential growth of this field. Although several reviews have been published on this subject, no systematic overview has been provided about applications exploring active targeting with emerging clinical

potential. Furthermore, none has focused solely on digestive tract carcinomas, an important subset of tumours with high incidence and mortality rates. Therefore, this systematic review will focus on active targeted nanodrugs for digestive cancers undergoing preclinical and clinical phases with particular emphasis on the ligands used for homing nanoparticles to cancer cells. We envisage raising awareness among the medical community on the potential of these anti-cancer agents and foster translational research on this field.

MATERIAL AND METHODS

Study selection

A systematic review was conducted through a MEDLINE database (PUBMED) search until October 2014, in order to retrieve papers that evaluate the therapeutic effect of nanoparticles loaded with conventional chemotherapeutic agents in digestive cancer tumors. All procedures were according to PRISMA guidelines [46].

The search strategy used the following query: ("Nanoparticles"[Mesh] OR "Liposomes"[All Fields] OR "Polymeric"[All Fields] OR "Inorganic"[All Fields] OR "Dendrimers"[All Fields] OR "Mesoporous silica"[All Fields]) AND ("Active"[All Fields] OR "Targeted"[All Fields] OR "Tumor targeting"[All Fields] OR "multifunctionalized"[All Fields] OR "Ligand"[All Fields] OR "Antibody"[All Fields] OR "Antibody Fragments "[All Fields] OR "Aptamers"[All Fields] OR "Glycoproteins"[All Fields] OR "Peptides"[All Fields] OR "Vitamins"[All Fields]) AND ("Antineoplastic Agents"[Mesh] OR "Doxorubicin"[All Fields] OR "Vincristine"[All Fields] OR "Paclitaxel"[All Fields] OR "Cisplatin"[All Fields] OR "methotrexate"[All Fields] OR "camptothecin"[All Fields]) AND ("drug delivery"[All Fields] OR "therapeutic use"[All Fields] OR "drug carriers"[All Fields] OR "drug effects"[All Fields]) AND (("stomach"[MeSH Terms] OR "stomach"[All Fields] OR "gastric"[All Fields]) OR colorectal[All Fields] OR ("colon"[MeSH Terms] OR "colon"[All Fields]) OR "rectal"[All Fields] OR ("pancreas"[MeSH Terms] OR "pancreas"[All Fields]) OR ("liver"[MeSH Terms] OR "liver"[All Fields]) OR

hepatic[All Fields]) OR ("oesophagus"[All Fields] OR "esophagus"[MeSH Terms] OR "esophagus"[All Fields])).

Inclusion and exclusion criteria

Only studies written in English were considered for inclusion. The following inclusion criteria were used: (1) Studies that used in vitro and in vivo models on digestive tumors; (2) Evaluation of the encapsulated chemotherapeutic drugs effect. Abstracts, reviews, letters, editorials and expert opinions were excluded.

Data extraction and collection

We assessed the studies using a hierarchical approach based on the title, the abstract and the full-text article. Two reviewers independently extracted the data from each study. Discrepancies between the two reviewers were resolved by discussion and consensus.

The digestive cancers evaluated were divided into four major categories, i.e., liver, colorectal, gastric and pancreatic tumors, based on tumor location. Then, the following data was extracted from each eligible study as follows (1) ligand; (2) receptor; (3) drug-NP; (4) animal model; (5) Outcome.

RESULTS

Selection of trials

A flow diagram of the literature search and study selection is presented in Figure 2. Applying the defined inclusion and exclusion criteria 43 studies were elected and organized based on the addressed tumour models, as summarized in Tables 1-4.

The majority of the selected studies focused on liver (51%) and colorectal (26%) cancer models, whereas only 19% and 7% addressed gastric and pancreatic cancer tumours, respectively. The most commonly used ligands were carbohydrates (34%), followed by antibodies or

antibody fragments (30%) and proteins or small peptides (32%). Even though a wide variety of chemotherapy drugs and nanoparticles were reported, the majority of the studies used DOX (trade name Adriamycin; 63%). The most explored drug nanovehicles were liposomes (53%) followed by biocompatible polymers (26%). These studies are discussed in detail in the following sections with emphasis on the ligands used for targeting.

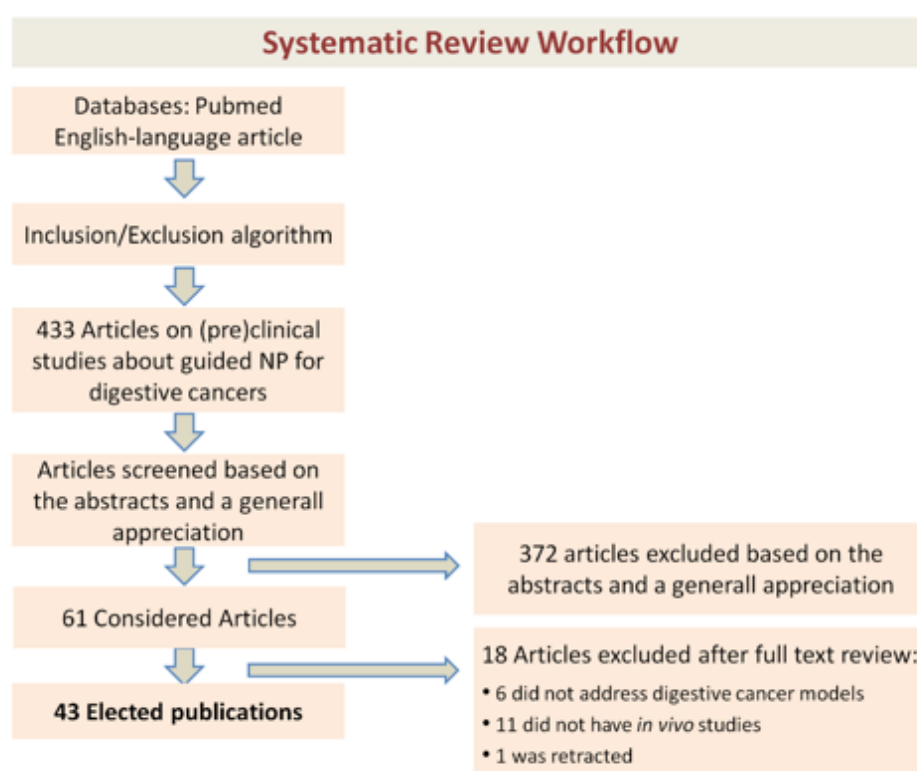


Figure 2. Flowdiagram showing the different phases of the systematic review.

Guided nanotherapies for Hepatocellular carcinomas

Hepatic guided-drug delivery systems have attracted much attention due to their high degree of selectivity to liver cells, enhanced uptake ability of drug-loaded nanoparticles into target sites, possible reduction of drug dosages and significantly decreased drug toxicity. According to Table 1, several studies explore the asialoglycoprotein receptors (ASGP-R1 and 2) which are exclusive and abundantly expressed by hepatic

parenchymal cells [47,48]. ASGP-Rs recognize and promote the endocytosis of desialylated plasma glycoproteins presenting glycosidic chains with terminal galactose and N-acetylgalactosamine residues [47,49]. Although ASGP-Rs are not specific of tumor cells, they are significantly overexpressed in both hepatocarcinomas and hepatocellular tumour cells [50,51], making them good candidates for active targeting. Nevertheless, some studies support that more advanced stage tumours may express lower levels of ASGPR1 when compared to the healthy liver tissue [52,53]. Furthermore, ASGPR1 has been associated with proliferative cells, that even though more susceptible to available cancer agents, may not represent subpopulations of more aggressive quiescent cells responsible by tumor relapse and dissemination [54]. Still, the strategy has allowed to increase the accumulation of anti-cancer agents in the liver, which is aided by the fact that liver physiology is conducive to the accumulation of NPs relying only in the EPR effect [55,56].

The most explored ligands for ASGP-R include galactose, galactosamine [38] and also lactose [57]. NPs functionalized with these molecules have shown higher anti-tumor efficacy and inhibition of the tumor growth than non-targeted nanodrugs and chemotherapeutic agents alone [47,49,58–63]. Namely, Cheng et al. (2012) showed that the administration of galactosylated functionalized chitosan NPs carrying 5-FU could inhibit by 75% the growth of tumours resulting from the orthotopic engraftment of 3×10^6 cells into athymic mice [64]. Moreover, the authors analyzed the apoptosis pathways and verified that active NPs upregulated p53 expression at both protein and mRNA levels with subsequent cell death pathway activation [64] (Figure 3). Noteworthy, is also the galactosamine functionalized N-(2-hydroxypropyl)-methacrylamide-DOX formulation (PK2, FCE28069). In preclinical studies, rats showed significantly fast blood clearance and high hepatocyte uptake of PK2, which was dependent on the molar percentage of galactosamine [65]. Reflecting the specificity of liver targeting, another study showed no accumulation of PK2 in colon carcinomas grown in the liver of athymic mice [53]. In the phase I clinical studies, a comparison between PK2 and

its non-guided counterpart PK1 showed that PK2 preferentially accumulated in the liver while PK1 did not demonstrated such accumulation effect [52]. However, PK2 accumulated less in tumor regions of the liver, which reinforces the immunohistochemistry studies associating malignant transformations with the loss of asialoglycoprotein receptors [66,67]. Still, the maximum tolerated dose (MTD) for PK2 in phase II was set at 120 mg/m², while the MTD of PK1 was 320 mg/m² [52,68], denoting the higher efficacy of the guided approach. Nevertheless, the absence of recent information in scientific articles and government databases involving PK2, suggests that it did not perform well in subsequent clinical studies. Altogether, targeting the ASGP-R may pose as a feasible strategy to deliver nanoencapsulated drugs to the liver. However, it will most likely benefit patients presenting tumors with a marked expression of the receptors, which are the early stage hepatic tumours. Also, a careful evaluation of ASGP-R expression in the tumours prior to the administration of the drugs is needed to determine the feasibility of such approach.

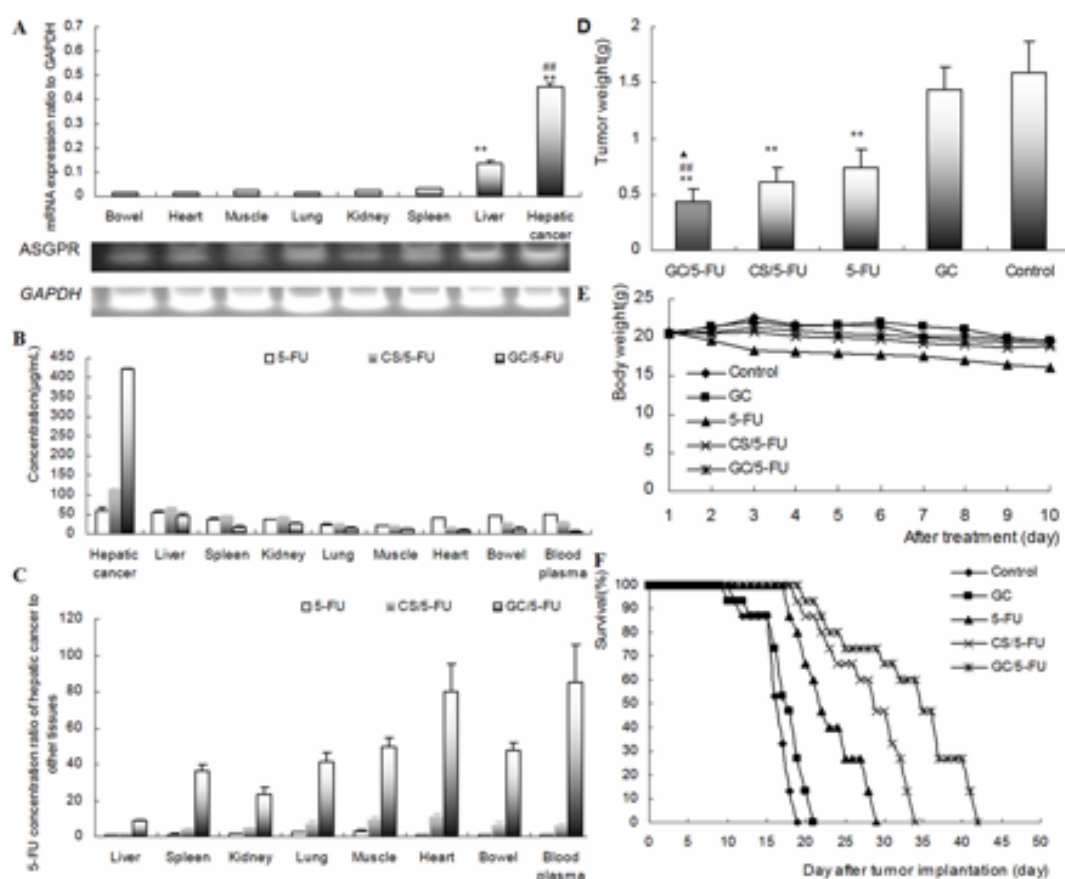


Figure 3. 5-FU nanoparticle had liver targeting and enhanced hepatic cancer growth inhibition through ASGPR in vivo. (A) Expression of ASGPR mRNA in different tissues of hepatic cancer, liver, spleen, kidney, lung, muscle, heart and bowel in the orthotopic liver cancer mouse model evaluated by RT-PCR. Results are shown as mean $\pm$ SD ($n=3$). (** $P < 0.01$, compared with spleen, kidney, lung, muscle, heart and bowel; ## $P < 0.01$, compared with liver). (B) 5-FU concentration in different mice tissues 30 min post i.v. injection of 5-FU, CS/5-FU or GC/5-FU. Results are shown as mean $\pm$ SD ($n=3$). Hepatic cancer tissue showed the highest 5-FU concentration, followed by liver tissue. (C) Ratio of 5-FU concentration in hepatic cancer cells in relation to other tissues. The 5-FU concentration in hepatic cancer tissue was 8.69-, 23.35-, 79.96- and 85.15-fold higher than in normal liver tissue, kidney, heart and blood, respectively. (D) Weight of liver tumors measured on Day 10. Results are shown as mean $\pm$ SD ($n=10$). Mice received GC/5-FU, CS/5-FU, 5-FU, GC or PBS on Day 5 after establishing the transplant model. ** $P < 0.01$ compared to control or GC group; ## $P < 0.01$ compared to 5-FU group, $\Delta P < 0.05$ compared to CS/5-FU group. (E) Body weight was monitored from Day 1 to 10 of treatment ($n=10$). Body weight decreased significantly more in the 5-FU group compared with the control, GC, CS/5-FU and GC/5-FU group. There was no difference in body weight between the GC/5-FU, CS/5-FU, GC and control groups. (F) Mice survival. The median survival in the control, GC, 5-FU, CS/5-FU and GC/5-FU group was 16, 17, 22, 29 and 35 days, respectively. The longest median survival time was seen in the GC/5-FU

group. (legend adapted from the original). Reproduced from reference[64]
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Another class of important ligands for active targeting against hepatic carcinomas include small proteins/peptides [37,69,70]. According to Table 1, NPs guided by small peptides such as HIYPRH, AGKGTPSLETT and RGD had strong and specific binding to the liver, enhanced uptake *in vitro*, and also promoted a retardation of tumor growth and prolonged survival times *in vivo*, when compared to non targeted NPs [37,69,71]. The HIYPRH (T7) peptide was used to co-delivery a therapeutic gene encoding human tumor necrosis factor-related apoptosis-inducing ligand (pORF-hTRAIL) and DOX to tumour-bearing xenografts. The T7 peptide was identified by phage display screening and showed an affinity for transferrin receptors similar to transferring itself [72] and its internalization was found to be facilitated by endogenous transferrin [73,74]. Transferrin receptors TFR1 (CD71) and TRF2 are overexpressed in hepatocarcinomas [75–77], most likely in response to iron deficiency during liver carcinogenesis [75]. Nevertheless, these proteins are also present in the healthy liver [78] and in erythroid precursors [79], raising an important toxicity issues that requires careful evaluation in the future. The homing peptide AGKGTPSLETT (A54), obtained by *in vivo* phage-display technology, was also found to specifically bind to human hepatocellular carcinoma cells, in particular the BEL-7402 cell line [80]. However, to the best of our knowledge, the receptor for the A54 peptide has yet to be identified. Accordingly, the A54 peptide was used as ligand on a PEGylated stearic acid grafted chitosan micelles loaded with DOX against BEL-7402 hepatoma cells [80]. The guided nanoconstruct showed higher affinity and internalization by human hepatoma cells than normal liver cells in co-culture. Furthermore this formulation presented higher distribution ability to liver and hepatoma tissues *in vivo*. Both *in vitro* and *in vivo* studies in mice reported an arrest in tumour growth and reduced toxicity compared with DOX [71]. Finally, the RGD homing peptide was used to deliver PTX-loaded PEGylated

PLGA-based NPs to hepatocarcinoma cells. The RGD peptide preferentially targets integrins $\alpha\text{v}\beta 3$ [81,82], which are highly expressed in angiogenic endothelial lining tumor and tumor cells, but poorly expressed by resting endothelial cells and most normal organs [83]. Integrin signaling regulates diverse functions in tumour cells, including migration, invasion, proliferation, survival and also plays a key role in angiogenesis [84,85]. Targeting the $\alpha\text{v}\beta 3$ integrin with drugs is expected to provide an opportunity to eliminate both tumour cells and the tumor endothelium and consequently destroy tumor vessels without significantly harming the microvessels of normal tissue [83,86]. In agreement with these observations, Danhier et al. (2009) demonstrated that RGD-grafted NPs showed higher affinity for Human Umbilical Vein Endothelial cells (HUVECs) by binding to $\alpha\text{v}\beta 3$ integrin in comparison to non-targeted NPs [69]. Likewise, RGD-guided NPs showed *in vivo* a high affinity for tumour vessels, decreased tumour growth and prolonged survival times in mice-bearing liver tumours when compared to non-targeted NPs. Altogether, RGD-labeled NPs against tumor vasculature has proven to be a promising approach against cancer, which has also been explored in other models, as discussed in detail in the following sections.

Conversely, only one study by Yuan et al, 2013, used the whole protein apolipoprotein A1 (Apo A1) to selectively guide a DOX HDL-encapsulated complex to the liver. This approach explored the fact that Apo A1 can mediate the delivery of cholesterol from peripheral tissues to the liver by binding specifically the scavenger receptor class B type 1 (SR-B1) receptor on the surface of hepatocytes. *In vivo* studies were conducted in athymic mice presenting hepatic tumours that resulted from the xenotransplantation of the LCI-D20 cell line. The authors reported that the 10 mg/kg dosage of rHDL-DOX was more effective in reducing tumor size than the corresponding control group (non-targeted NP), reducing tumor size by 44%. However, while the weight of the mice treated with non-guided drugs increased gradually, the weight of the mice treated with

guidance remained the lowest for the duration of the study, suggesting that the active targeting could translate in higher toxicity [87].

In addition to carbohydrate and peptide ligands, antibody-based guided nanotherapeutics has also demonstrated an improvement in tumor treatment and survival. As shown in Table 1, mAbs attached to different DOX-NPs, demonstrated a specific bind and consequently internalization of the nanocomplex by hepatic tumour cells *in vitro*. Moreover, mice xenografts administrated with guided drugs presented delayed growth of tumors in comparison to those exposed to non-guided and drug-free treatments [88–90]. Furthermore, imaging studies showed a reduced accumulation of the nanomedicine in other organs and less side effects [88,90], thereby confirming the specificity of the targeting strategies. Namely, Wang et al., developed an anti-CD44 antibody-mediated liposomes loaded with a suicide gene or a chemotherapy drugs that could specifically target the CD44⁺ hepatocarcinomas cells that could also be used for imaging. This approach is expected to allow targeting subpopulations of hepatocarcionoma cancer stem cells often responsible by drug resistance, tumour relapse and disease dissemination [91–93]. In another study, Roth et al. described a DOX-loaded immunoliposome designed to target the VEGFR-2 receptor, which is overexpressed by activated endothelial cells at angiogenic sites. The Fab' fragments of the monoclonal rat anti-VEGFR2 antibody DC101 was used as homing molecule. Of note, the DC101 has been found to block ligand binding and receptor signaling *in vitro* [94,95] and reduce, by itself, the neovascularization and produce antitumour effects in several human tumour xenografts [94]. However, the construct was also capable of targeting and deposit DOX in the vasculature of hepatocellular carcinomas and enhance the inhibition of tumours that stemmed from the subcutaneous injection of hepatocarcinoma cells into nude mice. Still, higher growth inhibition was observed for small tumours when compared to larger ones (>400 mm³), suggesting this approach could be more effective when delivered at a stage of early angiogenesis [89].

The folate receptor is overexpressed in several human tumors, including the liver tumors[96]. This potential target was explored by Maeng et al., (2010) [97], using the folate vitamin, a low molecular weight molecule, attached to a super oxide DOX-loaded NPs. The authors demonstrated that this nanomedicine allowed a higher drug accumulation and apoptosis in tumors cells when compared with free drug, and non targeted liposomes. Furthermore, reduced mortality without noticeable side effects was observed in animal studies [97].

Glycyrrhetic acid, an aglycone of glycyrrhizin, a natural triterpenoid extracted from *Glycyrrhiza glabra* (liquorice) root, has also been explored in the context of hepatic cancer guided nanotherapeutics. This molecule has antioxidant and detoxifying properties and has been shown to increase apoptosis in hepatoma cells [98,99]. Furthermore, it has high affinity to the liver [100–103] and was found to specifically bind to receptors on the liver cell membrane [104]. According to Guo *et al.*, glycyrrhizin-modified chitosan NPs loaded with DOX had significantly higher uptake into HEPG2 cells and cytostatic activity due to specific interactions between the ligand and receptor [10]. *In vivo* studies using cell line xenograft models demonstrated that guided NPs presented 80% higher tumor growth inhibition than the non targeted nanoparticle and free DOX controls. Furthermore, mice treated with guided NPs presented no weight reduction, which suggests that this approach reduces the side effects. Notwithstanding, a significantly lower amount of DOX was detected in the heart and kidney. Moreover, mice injected with active NPs did not die, whereas the mortality rate was 40% in group [10]. To our knowledge, this was the only study exploring a natural product as homing molecule to gastrointestinal cancer cells.

Altogether, the majority of the identified studies demonstrated that guided therapeutics improved the accumulation of the drugs in tumor cells and inhibited tumor growth in higher extent when compared to non-guided nanoconstructs and non-encapsulated drugs. Moreover, the majority demonstrated fewer side effects than the control groups. Interestingly, these observations were independent of the class of the

ligands used. Targeting hepatocyte-specific receptors such as ASGPR with carbohydrates has proven to be the most consistent approach to selectively target nanodrugs to liver, even when faced with the failure of PK2 in clinical trials phase. However, the interaction of the homing carbohydrates with endogenous lectins cannot be overruled and warrants careful investigation in the future in biodistribution studies. Exploiting small peptides and mAbs for homing to CD44, integrins and VEGFR has also been demonstrated. Given the pancarcinoma nature of these ligands, such solutions can be translated to other tumours. Again, all these approaches require a deeper understanding of the guided drugs biodistribution *in vivo*.

The drug-NP of election was DOX- liposome and 5-FU chitosan. The first approach is already approved by the FDA, with trade name Doxil® and exhibit fewer side effects than DOX alone [105]. Also several studies have shown that the therapeutic efficacy of 5-FU can be increased through binding the drug to carriers such as biodegradable nanoparticles, namely chitosan NPs [106,107].

Guided Nanotherapies for Colorectal cancer

Contrasting with hepatologic tumours, in colorectal models only one study addressed the potential of carbohydrates as cancer ligands. Guu et al., used galactosamine functionalized DOX-dextran conjugates against CT-26 colon carcinoma cells implanted in mice. Interestingly, DOX was covalently bound to the dextran via a tripeptide spacer (Gly-Leu-Gly) stable under circulation and susceptible to terminal cleavage by lysosomal enzymes following pinocytic internalization of the conjugate[108]. However, the guided nanotherapeutics showed no significant effect when compared to non-guided strategies, due to the fact that galactosamine preferentially delivers the drugs to the liver but not colon carcinomas.

Conversely, peptides were significantly explored as part of a strategy to destroy tumour associated vasculature. Recently a number of antibody-based therapies for colorectal cancer have been approved by the FDA.

Some examples include bevacizumab, a humanized antibody against VEGF, which associates with the angiogenic capability of the tumor, and was approved for metastatic colorectal cancer in 2004 [16,109,110]. Angiogenesis is a key process in the progression and metastasis of a [111,112]. This process comprises the construction of new blood vessels in tumor tissue, a critical process, since the supply of oxygen and nutrients is essential for tumor growth [112,113]. Several guided nanodrugs have also been developed and tested in colon cancer models against tumor vasculature (Table 2). However, in contrast to bevacizumab that targets VEGF, these nanosolutions were designed against tumour-associated endothelial cells. Five out of six studies used small peptides for homing nanodrugs to angiogenic sites, whereas one used an antibody. Namely, two studies from the Naoto Oku group explored the pentapeptide APRPG to deliver DOX-loaded PEG-coated liposomes. The APRPG peptide was identified by *in vivo* biopanning of a phage-displayed peptide library and found to bind specifically to VEGF-stimulated human umbilical vein endothelial cells [114]. Still, its exact ligand remains unknown. Animal studies in mice bearing the highly metastatic adenocarcinoma cell line 26 NL-17 [113] confirmed that intravenous administration of APRPG nanodrugs specifically targeted vessel-like structures leading to the apoptosis of both tumour endothelial and cancer cells [113,115,116]. In contrast, the non-guided nanoparticles only had effect against cancer cells [113,115,116]. However, the authors discuss that it is still unclear whether tumour cells are directly eliminated by APRPG-Lip-DOX or if apoptosis is promoted by a decreased in oxygen and nutrient supply. Subsequent studies demonstrated that DOX-loaded APRPG-liposomes could also significantly suppressed tumor growth in mice bearing DOX-resistant tumor P388 leukemia cells, suggesting that this drug delivery strategy may be beneficial to other types of tumours. In another study from the same group, a similar strategy was used, this time to study the effect of 2'-C-cyano-2'-deoxy-1- β -D-arabino-pentofuranosylcytosine (CNDAC), a synthetic agent that induces DNA strand breaks after its incorporation into tumor cells [117]. In line with the observations that

emerged from the studies involving DOX, 26 NL-17 xenografts benefited more from the treatment with APRPG PEG-coated CNDAC-loaded liposomes in comparison to those treated with non-target drug vehicles. In addition, these early reports contributed to the notion that PEG-functionalization improves the circulation half-life of the particles, resulting in enhanced accumulation in the tumor. Noteworthy, NPs were found to accumulate significantly in the spleen and liver [113,115,116]. Nevertheless, the author stated that the administration of both non-guided and guided particles promoted no alterations in the weight of the mice, denoting the lack of toxicity. Deeper investigations are warranted to fully disclose this possibility before engaging clinical trials phases.

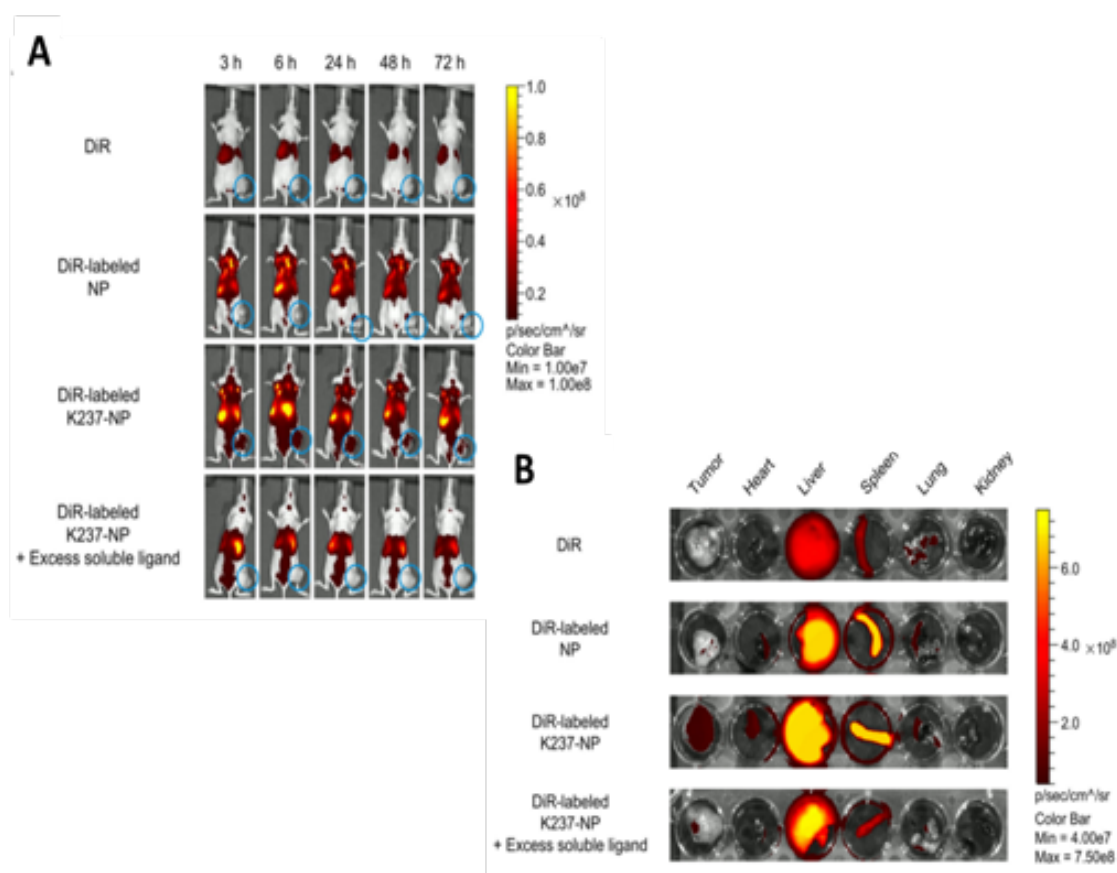


Figure 4. Representative (A) Whole body imaging of HCT-15 bearing female BALB/c nude mice under IVIS Xenogen after intravenous injection of DiR labeled nanoparticles. The blue circles indicate the tumors. (B) *Ex vivo* optical imaging of tumors and organs of HCT-15 bearing female BALB/c nude mice sacrificed at 72h after intravenous injection of DiR labeled nanoparticles. Yellow to dark red fluorescence represents high to low

fluorescence intensity, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article). Reproduced with permission from reference [118], **Copyright** (2013), with permission from Elsevier.

Bai et al also addressed the problem of multidrug resistance using the K237 (HTMYHHYQHHL) peptide-conjugated PTX loaded NPs against human colorectal adenocarcinoma HCT-15 xenografts (Figure 4). The HCT-15 cell line naturally expresses the drug-efflux pump P-glycoproteins and has been used as a drug resistance model [118–120]. The K237 peptide binds specifically and with high-affinity to VEGFR-2 receptors, overexpressed in the tumor vasculature, thereby competing with VEGF [121]. Furthermore, the peptide was found to exert a strong anti-angiogenic activity *in vivo* and inhibited the growth of breast tumours and their lung metastasis in mice [121]. Targeted NPs increased accumulation, induced the apoptosis of tumour endothelial cells, and promoted the necrosis of HCT-15 derived tumours in mice. More important, the authors reported that guided PTX provided an 8-fold increase in the anticancer effect when compared to the free drug combined with Tariquidar (XR9576), a potent P-glycoprotein inhibitor [121]. However, more studies are necessary to fully disclose the significance of these observations in the context of drug resistance. In particular, the introduction of multifactorial drug resistance models is important to disclose the capability of these solutions to overcome drug resistance.

The RGD-peptide, previously explored by other authors for anti-angiogenic drug targeting, was used by Schiffelers et al. to deliver DOX-loaded PEG-coated long-circulating liposomes to the DOX-insensitive murine C26 colon carcinoma model. The results from this study corroborate previous findings regarding the superior anticancer properties of targeted NPs [111].

More recently, Wicki et al. employed anti-VEGFR2 antibodies to target DOX-loaded PEGylated liposomes to endothelial cells in transgenic Rip1Tag2 mouse model of insulinoma, MMTV-PyMT mice model of breast cancer as well as in HT-29 human colon cancer mice xenografts. Again,

the immunoliposomes showed superior anticancer action when compared to the antibody alone and empty PEGylated liposomes and an effect similar to the one resulting from the oral administration of PTK387, a strong VEGFR1, -2, and -3 inhibitor [112]. In addition, the mice kidneys revealed no signs of acute toxicity three days after administration of the nanodrug. In addition weight monitoring and histological analysis of internal organs revealed no signs of long term toxicity. Interestingly, the administration of non-guided drugs led to an increase in tumour atypia, which was not observable for anti-VEGFR2-drugs, highlighting the higher toxicity of non-guided therapeutics. Despite the significant anti-angiogenic effect of anti-VEGFR2-PEG-Liposomes, the response of the blood vessels was dependent both on their localization and morphology. While the guided strategy preferentially removed tumour-associated vessels, the healthy ones remained unaffected, most likely due to the differential expression of VEGFR2 between quiescent and tumor-associated vasculature [112]. Also, capillaries presenting a single layer of endothelial cells were more susceptible to therapy while endothelial cells within vessels protected by smooth muscle cells were less responsive to therapy. The authors further discuss that anti-VEGFR2-liposome-DOX was more effective in Rip1Tag2 mice than classical chemotherapy and other ongoing experimental treatments [112].

In resume, all the above described strategies consensually demonstrated the anticancer potential of guiding nanodrugs to tumour vasculature. The enhanced therapeutic efficacy of this approach has been attributed to the synergism of several aspects which include: i) destruction of neovessels by damaging angiogenic endothelial cells, which leads to a cutoff in the oxygen and nutrients supply to the tumor; ii) increase of the concentration of anti-cancer agents in the tumour due to the EPR effect. Altogether, these events may provide a strategy to overcome the problems posed by drug resistance and promote tumour necrosis with less toxicity than the free drug. Noteworthy, several studies indicate that tumour vasculature may develop by VEGFR-independent signaling pathways [122], that may account for the resistance to anti-

angiogenic drugs directed solely to VEGFR. Even though promising against the tumour bulk, antiangiogenic drugs may be ineffective against hypoxic niches that harbor more aggressive cancer cells, endowed with stem cell properties, chemoresistance and capability to migrate and promote metastasis [123–125]. In addition, it may, by itself, contribute to the formation of such hypoxic niches and, consequently, the development and maintenance of highly malignant subpopulations. In this context, the co-administration of drugs specifically targeting hypoxic cells and/or molecules capable of interfering with the HIF-1 α /2 α signaling pathways, which are the main promoters of hypoxia-associated transformations [126,127] may pose as interesting solutions to be evaluated in future studies.

The remaining five studies concerned strategies against tumour cells. The first was an early study, presented almost 20 years ago, involving the AR-3 mAbs, an IgG1 that recognizes the CAR-3 antigen, widely expressed on human adenocarcinomas of the stomach, colon, and ovary [128–130]. The antibody was successfully used to guide liposomes carrying two derivatives of 5-fluorouridine (5-FUR), a highly cytotoxic metabolite of 5-FU, to colon cancer cells [131]. The other more recent study stemmed from a roadmap established by Hosokawa et al. to identify highly specific cancer antibodies with potential clinical applications, including target drug delivery. Briefly, human mAbs were obtained from hybridomas resulting from the fusion of mouse myelomas with lymphocytes isolated from regional lymph nodes from cancer patients with different tumours. This approach was based on evidences that cancer patients can mount a humoral response against tumour-associated antigens [132,133]. It led to the identification of the GAH antibody that specifically recognizes and is internalized by colon and gastric cancer cells, but not the corresponding healthy tissues. Subsequent reports identified the human non-muscle myosin heavy chain type A as the GAH epitope [134]. Interestingly, this is a cytoskeletal component and not a cell surface protein, raising questions about the mechanisms by which it is exposed on the cell surface allowing recognition by GAH and

internalization. Following these observations, Hamaguchi et al. demonstrated by immunohistochemistry that 94% (100/106) of surgical specimens of colorectal tumours expressed GAH. Furthermore, these authors developed a PEG-tagged DOX-loaded liposome functionalized with the F(ab')₂ of GAH (MCC-465) that showed significant anticancer activity in comparison to the pegylated liposome DOX (PLD) against GAH-positive WiDr-Tc and SW837 xenografts but not against GAH-negative Caco-2 xenografts. The MCC-465 will be discussed in further detail in following sections, since it followed through to phase I clinical trials against gastric cancer.

Also, Lee et al. explored lipids as homing molecules. The cationic lipid 3,5-dipentadecyloxybenzamidinium hydrochloride (TRX-20), with high affinity for chondroitin sulfate (CS), was used to deliver cisplatin-loaded polyethylene glycol (PEG)-coated liposomes to different types of tumour models. Namely, the nanodrug was tested in vitro and in mice xenografts against highly metastatic ACHN human renal adenocarcinoma, LM8G5 murine osteosarcoma and HT-29 human colon adenocarcinoma cell lines. The immunoliposome showed preferential binding and internalization by ACHN and LM8G5 cells, which were found to overexpress CS at the cell surface. In contrast, it showed little affinity to the human colon adenocarcinoma cell line used in this study, due to its marginal levels of CS. TRX-20 liposomes also showed significant anti-cancer activity against CS-expressing cells ACHN and LM8G6 in vitro, whereas non-guided liposomes were ineffective. Animal tests showed that guiding doubled the accumulation of the drug in tumours expressing CS in comparison to PEG liposomes. Furthermore, it provided a higher reduction in tumour local growth, prevented metastasis to the liver and increased mice overall survival when compared to non-guided therapeutics and the free drug. However, it was not effective against colon cancer cells that did not express high levels of CS. Still, the growing insights on the structure of colon cancer-associated glycosaminoglycans [135] may now pave the way for more specific ligands towards more effective nanodrugs.

More recently, Jalalian et al. presented the first study involving the use of aptamers in guided nanotherapeutics for digestive track tumours. Aptamers are oligonucleotide sequences with high affinity for target ligands, that present reduced immunogenicity, good tumour penetration, rapid uptake and clearance when compared with their monoclonal antibody counterparts [136,137]. Furthermore, aptamers offer a more easy scale up production and facilitate the implementation of good manufacturing practices in comparison to moAbs [136,137]. In this study the 5TR1 DNA aptamer was applied to target abnormally glycosylated forms of mucin-1 (MUC-1), a densely O-glycosylated and high molecular weight cell-surface glycoprotein [138]. This strategy explores the fact that malignant transformations are often accompanied by profound alterations in the cells O-glycosylation machinery, which translates into the formation of unique protein glycovariants at the cell surface that are not present in the corresponding healthy tissues [139,140]. These alterations are amplified by the overexpression of MUC-1 in several epithelial tumours, including pancreas [141], colon [142] and gastric carcinomas [143], thereby providing means for highly selective targeting [144]. Contrasting with previously described studies exploring mostly liposomes and other bioorganic materials as encapsulating agents, Jalalian et al. used super paramagnetic iron oxide nanoparticles (SPIONs) loaded with EPI. Due to their EPR effect and magnetic resonance traceability, SPIONs enabled both target-specific cancer therapy and Magnetic Resonance Imaging. Furthermore, the EPI-433 Apt-SPION complex could significantly reduce the tumor growth in C26 murine colon carcinoma cell xenografts when compared with EPI alone [138].

In summary, more than half of the pre-clinical studies involving colorectal cancer models have been directed to tumour neo-vasculature, using mainly small peptides for homing. The remaining studies used monoclonal antibodies, small peptides, lipids and also aptamers for different types of cancer cell surface molecules. In agreement with the observations stemming from other models, all reports demonstrated the higher therapeutic efficacy of guided therapeutics in comparison to non-

guided approaches and the free drug, irrespectively of the used ligand. However, to the date no solution has transposed to clinical trials phase. The drug of election was DOX, used in colorectal cell line xenografts, whereas liposomes were the main delivery vehicles.

Guided nanotherapies for Gastric cancer

Few studies have been conducted involving guided nanotherapeutics for gastric cancer (Table 3), of which three explore peptides/proteins and other three use either whole antibodies or antibody fragments for homing.

In particular, two peptide-based solutions have been developed to address peritoneal dissemination, which poses as an independent predictive factor of poor prognosis and a major therapeutic challenge. Over ten years ago, Iinuma et al used a PEG-modified liposome conjugated to transferrin to deliver cisplatin to nude mice with peritoneal disseminated tumours resulting from the injection of MKN45P human gastric cancer cells. These cells were found to overexpress TFR and EGFR on the surface. According to the study, mice that received intraperitoneal TF-PEG liposomes presented high concentrations of cisplatin in the peritoneal cavity and in tumour cells overtime as well as enhanced life expectancy than those administrated with PEG-liposome, bare-liposome or free cisplatin. Contrasting with PEG-functionalized liposomes, bare liposomes disappeared rapidly from the peritoneal cavity and peripheral blood circulation and accumulated in the liver and spleen, most likely a result of uptake by reticuloendothelial system [145]. Altogether, TF-PEG liposomes presented two main advantages: i) resistance to reticuloendothelial system by PEG modification, avoiding rapid clearance from the blood stream, namely by macrophages; ii) high accumulation in tumour cells by binding to the Tf receptors, displayed on the surface of tumour cells [146]. However, recent studies have brought to light that gastric adenosquamous carcinomas stem cells, responsible by drug resistance, disease relapse and often progression, are characterized by

the lack of CD71 transferrin receptor [147]. Therefore, despite the significant overexpression of CD71 in several types of tumours [148–150], these findings reinforce the need for a deeper evaluation on the context of TFR expression in gastric cancer to foster targeting approaches based on this receptor.

Also, Akita et al. identified a KLP tri-peptide motif with homology with laminin 5 (a ligand for $\alpha 3 \beta 1$ integrin) and high affinity to peritoneal tumours. The conjugation of the peptide SWKLPPS containing the KLP motif with liposomes bearing DOX improved the accumulation of the nanoparticles in tumours and enhanced the anti-tumour activity in relation to avoid liposomes. Altogether, the peptide motif KLP also presents potential to improve the nanodrug-based treatments of peritoneal metastasis of gastric cancer [151].

In another study, Chen et al. developed single and multi-target liposomal nanodrugs for gastric cancer treatment. The authors used the somatostatin analog octreotide alone or in combination with the RGD peptide, to selectively direct PEG-liposomes carrying dihydrotanshinone to gastric tumours and tumour neovasculature [152],[153]. Somatostatin is a peptide hormone, produced in the gastrointestinal tract, which suppresses growth hormone (GH), thyrotropin (TSH), insulin and gut hormone release affecting multiple aspects of GI function [154,155]. Somatostatin analogs, such as octreotide are well-established treatments for tumors that over secrete these hormones, including gastric endocrine tumors associated with autoimmune chronic atrophic gastritis[156]. These molecules promote their effect by selectively binding somatostatin receptors (SSTR), a family of five G-protein coupled membrane receptors, abundantly expressed in the gastric niche [155,156]. In the context of guided therapeutics, the presence of these receptors also in gastric carcinomas offers potential for active target drug delivery [157,158]. However, octreotide was more effective against moderate differentiated SGC7901 gastric cells than poorly differentiated AGS and MKN45 cells that did not express the target receptor. In addition, there have been reports of a decreased expression of somatostatin receptors expression

in more advanced stage gastric tumours in relation to early lesions and healthy tissues [159]. These observations suggest that this strategy would better serve to home drugs to initial tumours rather than more advanced stage cancers. Regarding the encapsulated drug dihydrotanshinone I, a lipid-soluble molecule isolated from Danshen, the root of Chinese herb *Salvia miltiorrhiza*, has been associated with several health benefits including anti-tumor activity [160,161]. Dihydrotanshinone has been reported to inhibit topoisomerase activity [162] and to promote apoptosis of leukemia cells [163]. Nevertheless, its mechanism of action is not yet fully understood. According to Chen et al., nanoencapsulation enhanced the anti-cancer properties of this natural compound in comparison to the free drugs. This effect was more pronounced for the multitarget approach both in vitro and in animal tests in AGC cell nude mice xenografts. Furthermore, the treated animals showed no significant signs of toxicity, translated by body weight evaluation [152]. To our knowledge this is the first work demonstrating the potential of a multitarget approach in relation to single-target solutions.

In addition to peptides/proteins, early studies by Goren et al. have explored the N-12A mAb to target DOX-loaded PEG-liposomes to erbB-2 (Her/2) expressing gastric cancer cells. The immunoliposome showed in vitro specific binding to erbB-2 positive N-87 human gastric cancer cells, however without noticeable cytotoxicity. Animal tests in nude mice presenting subcutaneous engraftments of the cell line showed no differences between antibody-conjugated and plain liposomes regarding liver and spleen uptake, accumulation in the tumour and anti-cancer activity. However, liposome encapsulation increased the therapeutic efficacy of DOX in comparison to the free drug. Accordingly, the authors suggested that the effectiveness of the described construct was not related with its affinity to target cancer cells; nevertheless, its internalization by cancer cells was not evaluated to disclose the influence of the antibody [164].

In another early study Uyama et al., developed an immunoliposome to deliver DOX to gastric cancer tumours. The murine monoclonal

antibody 21B2 F(ab')₂ fragment against human carcinoembryonic antigen (CEA), highly expressed by gastric tumours [165,166], was used for guidance. A comparison between F(ab')₂ and whole antibody functionalized immunoliposomes in MKN-45 gastric cancer cell line mice xenografts showed that the former led to a higher accumulation of the drug in the tumour, in accordance with previous reports for different models [167], revised by Sapra et al [168]. The administration of guided therapeutics also translated in higher anti-tumour effect when compared to non guided-liposomes and the free drug.

The MCC-465 agent, described in detail in the colorectal cancer section, has also been applied successfully in gastric cancer target therapeutics against B37 stomach cancer cell line xenografts. Three weekly doses of guided nanoliposomes carrying 3 mg DOX/Kg, which is nearly the maximum tolerated dose of free drug [169], strongly inhibited tumour growth without significant change in mice body weight. Conversely, the administration of non-guided liposomes and the free drug showed no significant effects, reinforcing the notion that targeting encapsulated drugs is determinant to improve their action. Following promising pre-clinical results, the MCC-465 was administrated to patients with metastatic or recurrent stomach cancer in phase I clinical trials [170]. Even though no anti-tumour response was observed, 10 out of 18 evaluated patients showed signs of disease progression. Furthermore, MCC-456 was well tolerated. The recommended dose for phase II, for a 3-week schedule, was set at 32.5 mg/m² in an equivalent amount of DOX.

In resume both peptide and antibody-based guided therapeutics have been developed to deliver drug loads to gastric tumours and peritoneal disseminated disease (Figure 5).

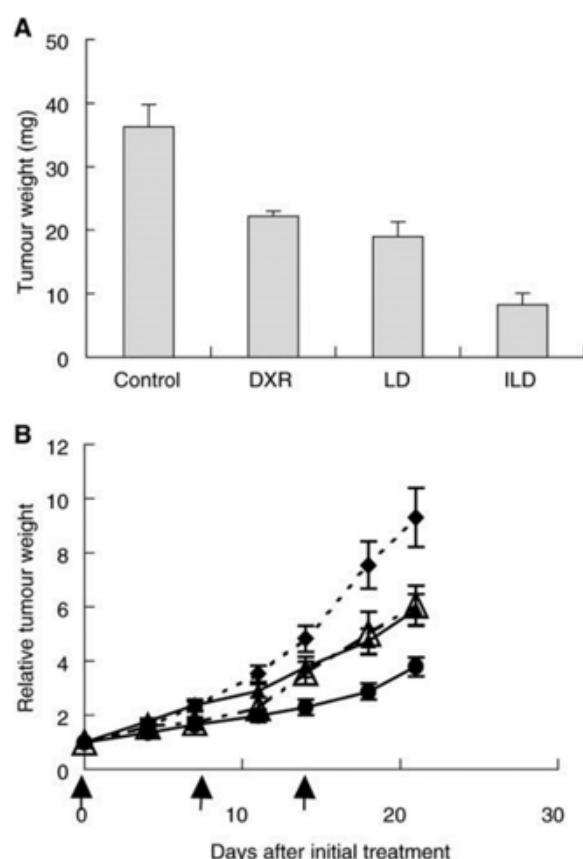


Figure 5. Representative comparison of therapeutic effects of ILD (imunoliposomes), LD (liposomes) and free DXR (doxorubicin). On SRC models (A), ILD, LD or free DXR was injected three times i.v., with 3.0 mg kg^{-1} in terms of DXR, into nude mice ($n=6$) bearing MKN45. The antitumour effect of ILD was significantly higher than that of LD ($P<0.05$) or DXR ($P<0.01$). Bars: means $\pm$ s.e. MKN45 cells implanted in SC (B) were treated with LD (▲, $n=13$), ILD (●, $n=12$), free F(ab')₂/GAH (0.36 mg kg^{-1}) mixed with LD (△, $n=6$), at a DXR equivalent dose of 2.2 mg kg^{-1} , or saline (◆, $n=12$), by i.v. injections on the 3 days indicated (m). Change in tumour weight was determined over the treatment period. The antitumour effect of ILD was significantly higher than that of LD ($P<0.05$) or LD supplemented with free GAH ($P<0.05$). Bars: mean $\pm$ s.e. Reproduced with permission from reference [134], Copyright (2003), with permission from Cancer Research UK.

In agreement with previous observations for other gastrointestinal models, all studies demonstrate the enhanced anti-cancer effect and lower toxicity of targeted solutions in comparison to non-target strategies and the free drugs. However, MCC-456 was the only one progressing to phase I clinical trials. The absence of reports regarding this drug associated with its lack of significant anti-tumour response suggests that

the use of this nanocomplex may have not progressed to phase III clinical trials.

Guided nanotherapies for Pancreatic cancer

Even with optimal surgery and aggressive adjuvant chemotherapy, the 5-year survival rate of pancreatic cancer patients does not exceed 20% of the cases [171,172]. Furthermore, less than 20% of patients are eligible to surgery due to disseminated disease [173]. In addition, pancreatic cancer poses significant problems for drug delivery due to its high desmoplastic nature [174]. However, our query only identified three studies exploring guided nanotherapeutics for pancreatic tumours (Table 4).

Namely, Murphy et al. developed RGD-peptide mimetic cRGDfK (f denotes D-phenylalanine) functionalized lipid-coated nanogels, with a human serum albumin core, loaded with taxanes (plaxitel and docetaxel). This molecular construct was used to target pancreatic tumour neovasculature via $\alpha\beta3$ integrins. It combined surface functionality provided by liposome with the advantages of Abraxane (PTX bound human albumin nanoparticles) regarding the stable drug loading of hydrophobic drugs. NPs using $\alpha1$ -acid glycoprotein as a core were also developed. The rationale underlying this choice was that both core proteins had been found to bind cancer drugs in circulation³². The gel core, formed by photo-crosslinking between these proteins and the lipid bilayer, enhanced the encapsulation of drugs with distinct physicochemical properties, namely PTX, docetaxel, bortezomib, 17-AAG, sorafenib, sunitinib, bosutinib, and dasatinib. This may also overcome one of the major limitations posed by liposomes, micelles, and block copolymers regarding the delivery of chemotherapeutic agents that do not present a weak base such as DOX. These findings suggest that a careful tailoring of the core may allow the stable delivery of different types of drugs to tumours. Guided nanogels loaded with PTX or docetaxel were then compared to Abraxane (bound albumin nanoparticles) in the

orthotopic R40P pancreatic cancer mouse model [81,175]. The administration of taxane-loaded nanogels led to a 40% decrease in tumour burden and produced a 15-fold improvement in anti-tumor activity in relation to Abraxane, by blocking both primary tumor growth and spontaneous metastasis, again reinforcing the benefit of active targeting [175].

MAbs have also been used to target drugs to pancreatic cancers. Camp et al. applied the SGT-53 therapy to target cancer cells via the transferrin glycoprotein receptor, which is ubiquitously and highly expressed on tumor cells as well as efficiently internalized [176–178]. SGT-53, a cationic liposome encapsulating a plasmid encoding human wild-type p53 DNA, was used to also co-deliver gemcitabine. The liposome was functionalized with an anti-transferrin receptor (TFR) scFv that, due to its smaller size in comparison to the whole antibody, is expected to have higher penetration into solid tumours [148,179]. The fact that these antibody fragments are recombinant proteins enables large scale production and strict quality control enabling clinical translation. In vivo studies, using an immunocompetent metastatic pancreatic cancer mice model, showed that p53 restoration in combination with gemcitabine, promoted DNA damage and apoptosis activation. Moreover, it was observed an extended life span, decreased tumour burden and increased cell apoptosis [148]. Taking into account the encouraging phase I results of SGT-3, comprehending solely gene therapy [180], the authors envisage Phase Ib/II clinical trials for pancreatic cancer involving the combination of these therapies.

The second study by Qian et al. describes a target vector that made possible the systemic administration of Arsenic trioxide (AS), a highly toxic anticancer drug [181–183], by minimizing its side effects. An arsenite-loaded maleimide amphiphilic diblock copolymer of poly (ethyleneglycol) and poly (d, l-lactide) construct, termed mal-PEG-PDLLA, was functionalized with and an anti-CD44v6 single chain variable fragment (scFvCD44v6). The ScFvCD44v6, screened out from the human phage-displayed scFv library, demonstrated higher specificity and affinity

than the full length antibody to CD44v6, over-expressed not only in pancreatic but also in hepatocellular [184], colorectal [185] and gastric cancers [186] and often associated with cancer stem cell phenotypes [187,188]. The ScFvCD44v6 includes variable domains of the heavy and light chains connected by a flexible peptide linker [189]. Animal tests involving the mouse model bearing subcutaneous human PANC-1 xenografts showed that scFvCD44v6-AS-NPs presented a higher accumulation in the tumor site than the non-guided nanoparticles and inhibited tumor growth by enhancing cell apoptosis (Figure 6). In addition, mice treated with the guided NPs also showed an increase in body weight during the course of treatment similar to the controls, suggesting lack of toxicity of the NPs. Furthermore, the administration of the free drug as led to a significant loss of body weight and four out of six mice showed severe adverse side effects, which included fatigue and crooked spine [190]. Noteworthy, the free drug treatment showed significant therapeutic effects in vitro that were not sustained in vivo, which may be explained by the poor accumulation of the drug in tumour tissues due to the lack of EPR effect provided by nanoencapsulation, leading to a faster elimination by the renal system [191]. Based on these and other observations, antibody fragments allow increasing the number of active ligands that can be linked to the NPs surface, present higher affinity for the ligands, greater biophysical stability and lower immunogenicity in vivo when compared to the full length antibody [192,193]. Adding to this, their small size may increase penetration into the tumour, improving the efficacy of anticancer drugs [reviewed by [158]].

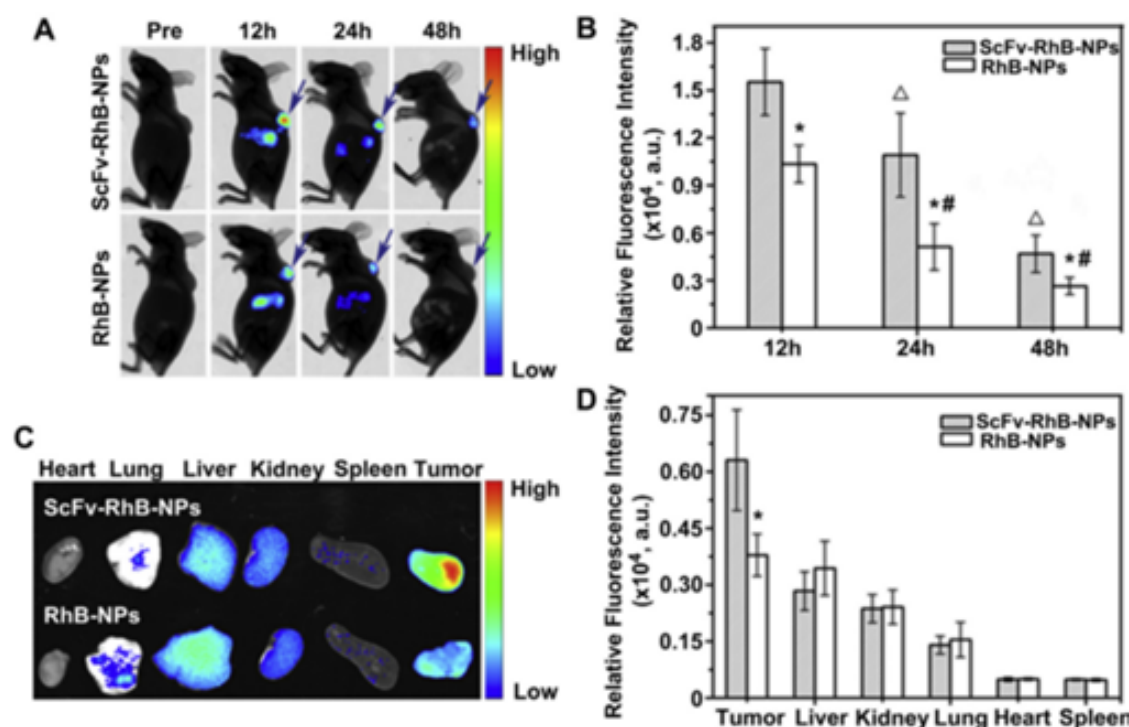


Figure 6. Representative *in vivo* and *ex vivo* fluorescence images of mice bearing PANC-1 tumor. (A) Whole-body images of tumor-bearing nude mice administrated with RhB-NPs and scFv-RhB-NPs at 12h, 24h and 48h after injection through tail vein respectively. (B) The fluorescence images were overlaid with X-ray images of the mice using Carestream MI software. The colour bar (from red to blue) indicates the change of fluorescence signal intensity from high to low. Quantification of the fluorescence intensity at tumor site at 12h, 24h and 48h *in vivo*. (C) Fluorescence images of the excised organs and tumors acquired at 48h post-injection. (D) Quantification of the fluorescence distributed in the organs after 48h injection. Results were expressed as the mean $\pm$ SD (n=3). *P < 0.05, vs the scFv-RhB-NPs group; Δ P < 0.05, vs the scFv-RhB-NPs group at the 12h time point; #P < 0.05, vs the RhB-NPs group at the 12h time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Reproduced with permission from reference [190], Copyright (2013), with permission from Elsevier.

Altogether, all three described strategies used to target nanodrugs to the pancreas are also applicable to other gastrointestinal tumours that also express the same ligands. Like previously described for liver, colorectal and stomach carcinomas, guidance also improved the retention and the anti-pancreatic cancer properties of non-guided nanodrugs, with fewer side effects *in vivo* using xenograft models. These effects are

particularly pronounced when compared to non-encapsulated drugs. Nevertheless, despite exciting preliminary pre-clinical findings significant efforts should now be put into the translation of these observations to clinical studies. Again, we emphasize the need for a deeper understanding on the biodistribution and bioaccumulation of guided therapeutics in vivo and its implications overtime.

Concluding Remarks

A plethora of exciting data has emerged from the studies identified by our systematic review about the anticancer potential of guided nanotherapeutic agents against digestive cancers. One of the main strategies targeted VEGFR2 or $\alpha v \beta 3$ integrins on tumour-associated endothelial cell surface receptors (Figure 7), either with small peptides or mAbs. The other strategy was based on targeting malignant cells in the tumour (Figure 7) and, in addition to small peptides and mAbs, carbohydrates, whole proteins, lipids, vitamins, natural products and aptamers were also explored. Irrespectively of the type of ligands used for homing, the molecular nature of the drug vehicle or the chemotherapy agent, all studies have consensually demonstrated that guidance potentiates the anticancer properties of nanoencapsulated drugs. Other important aspect is the lower toxicity evidenced by guided drugs in comparison to non-guided and conventional agents, which is critical for elderly population, which constitute the majority of digestive cancer patients. It is likely that the synergism of the EPR effect, provided by the nanodimensions of the drug carriers, associated with high affinity binding to tumour cells, conferred by the ligands, may contribute to these observations [193,194] (Figure 7). However, the role played by the ligands in the context of nanotherapeutics is still controversial, as some authors supported that the homing molecules only provide increased intracellular uptake of the nanocomplex [195,196], while others described that the ligand plays a significant role in tumor tracking [197,198]. Supporting some of these observations, Maeda and colleagues described that the

ligands did not improve the accumulation of the nanomedicine in the tumor, but instead it increased the therapeutic effect of the drug [116]. Also Hussain et al. and Kirpotin et al., showed that targeted and non-targeted immunoliposomes had the same pharmacokinetics and biodistribution, however guided nanoparticles accumulated predominantly within tumor cells, whereas the non-guided ones accumulate in the tumor stroma and extracellular space[195,199,200].

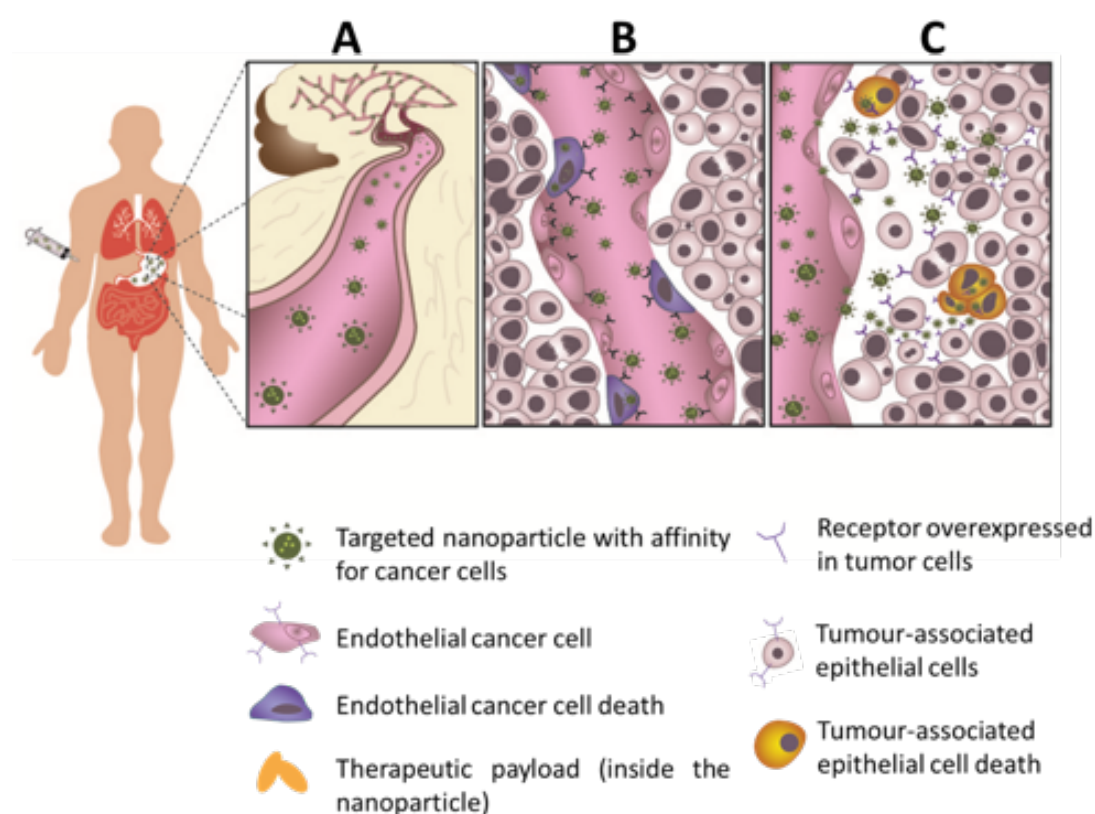


Figure 7. Representation of the two most common types of guided nanotherapies for digestive tumours (anti-angiogenic and anti-tumour cells). (A) Guided nanodrugs carrying chemotherapy payloads passively accumulate in tumour sites based on the EPR effect (described in detail in Figure 1) and also actively target specific cancer cells based on the affinity to cell-surface receptors overexpressed by cancer cells. The synergism of these events enhances the therapeutic effect of target medicines in relation to non-target approaches. The guided nanomedicines bind to cell-surface receptors, experience internalization and, once inside the cell, induce cell death, while the receptor is recycled back to the cell-surface. The release of the drug inside the cell is triggered by different physiological events, which may include sensitivity to pH, salinity, exposure to proteolytic enzymes or may be triggered by external stimuli such as radiation or

temperature. On the other hand the encapsulation agent may spontaneously dissociate as a consequence of a time-dependent degradation. (B) Guided Nanoparticles for tumour-associated endothelial-cells promote the selective destruction of the vessels responsible by feeding the tumour. (C) Guided Nanoparticles for tumour cells penetrate throughout the tumour and selectively bind to tumour cells expressing the ligand of interest, promoting their death.

More in depth studies based on the biodistribution and pharmacokinetics of each construct are clearly needed to fully elucidate how targeting influences treatment outcome, and also to disclose the impact of the ligand, the composition of the vehicle and the type of drug. Noteworthy, small peptides resulting from phage display libraries were among the ligands of election, either in drugs for tumour vasculature as well as for tumour cells. Small peptides combine high affinity binding to receptors and low immunogenicity with an easier and less expensive scale up production, when compared to more complex ligands such as monoclonal antibodies, enabling the translation of these solutions to clinical settings [18,201].

While anti-angiogenic strategies have shown remarkable anticancer potential, it also poses a significant risk related with the promotion of hypoxic niches that remain to be addressed and overcome. As discussed in detail in the colorectal cancer section, the administration of such drugs may favour the development of more chemo-resilient clones endowed with stem cell and mesenchymal properties, ultimately leading to invasion and metastasis. Notably, none of the studies presented so far on this subject have provided information on the molecular nature of the tumour populations resulting from the exposure to guided nanodrugs. Likewise, the molecular and functional characterization of chemoresistant populations were also absent from reports concerning drugs designed for tumour cells. Nevertheless, even though most anti-angiogenic studies were conducted for colorectal cancer, this is a pancarcinoma approach with potential application in the majority of solid tumours.

Regarding the strategies focusing on solid tumours, we concluded that glycans such as galactose and glucosamine, recognized by ASGPR,

are the ligands of choice to target the liver. In contrast to other homing molecules, this was the only strategy that cannot be translated to other digestive track models, since the target receptor is exclusively expressed by hepatic parenchymal cells. Nevertheless, the quenching of glycan-guided nanotherapeutics by endogenous listings is not to be overruled and also warrants careful evaluation in the future. On the other hand, the decreased expression of ASGPR in more advanced stage tumours, normally presenting high resistance to chemotherapy, when compared to the healthy liver is well documented[50,51]. These observations may, in part, account for the lack of encouraging results presented by the PK2 drug in phase I clinical trials. Nevertheless, the notion prevails that specific guided drugs to the liver may be achieved using this strategy and may serve patients overexpressing the ASGPR receptors. Ligands such as the GAH antibody [202] have also been proven useful against colorectal and gastric cancers, as translated by the election of MCC-465 for clinical trials [170]. Again, and despite somehow disencouraging phase I results for metastatic gastric cancer, the GAH antibody holds potential for more than 90% of gastric tumours, vowing for its exploitation in future studies. Another important nanocomplex undergoing clinical evaluation is MBP-426, a transferrin receptor-targeting liposome containing oxaliplatin. Currently MBP-426 is in phase II trials as second line treatment for gastric, gastroesophageal and esophageal adenocarcinomas [203].

Nevertheless, we note the few existing studies for gastric and pancreatic tumours in comparison to hepatocarcinomas and colorectal tumours and the absence of pre-clinical studies involving esophageal tumours. More surprisingly, although significant efforts have been put in pre-clinical development phases, only three guided nanomedicines have progressed to phase I clinical trials and none was yet been approved. This is in clear contrast with the promising results obtained with non-guided nanosolution such as Abraxane against gastric tumours [31] and Zinostatin, a neocarzinostatin-encapsulated styrene maleic acid nanocopolymer, that was clinically approved for unresectable hepatocellular carcinoma [35]. The fact that clinical studies conducted so

far with guided nanotherapeutics involved only late stage metastatic patients may, in part, account for these findings. We also note that the molecular nature of the metastasis may be different from the corresponding primary tumours [204]. Therefore, therapeutics tailored to target the main tumour may fail to avoid disease dissemination and poor outcome.

Even though our review focus on target chemotherapeutics, it is also important to mention two ongoing phase I clinical trials involving targeted gene nanotherapies, CALA-01 [206] and SGT-53 [207]. These drugs have been designed to bind transferrin receptors and offer potential against gastrointestinal tumours that overexpress this protein. CALA-01 was the first targeted, cationic cyclodextrin-based polymer-based nanoparticle carrying siRNA to be systemically administered to humans [208]. It allowed reducing the expression of the M2 subunit of ribonucleotide reductase, which is a relevant cancer agent [209][210]. The SGT-53 nanocomplex was able to deliver p53 DNA to cancer cells, which improved drug resistance associated with the loss of *p53* in pre-clinical settings [209][210]. Noteworthy, the co-administration of SGT-53 and gemcitabine to pancreatic tumours has significantly improved pancreatic cancer treatment in preclinical settings [207]. Such observations suggest that the combination of chemotherapy agents and gene therapy *via* targeted nanovehicles may allow improving cancer treatment. Nevertheless, this remains mostly an unexplored anti-cancer strategy.

Another important aspect is that few solutions have been specifically designed for chemoresistant cells. A more complete understanding of the molecular nature of these clones and metastatic cells allied with the identification of highly specific cell-surface biomarkers by proteomic approaches is needed. We believe that it will help speed the translation of active targeting therapeutic into clinically relevant molecules.

Some limitations of the studies discussed in this systematic review concern the heterogeneity of the cell lines used for each tumour model, which hamper accurate comparisons between reports [211]. Also, animal studies have been conducted in rodents, which in contrast to humans,

present fast growing tumours with leakier vasculature, thereby contributing to an enhanced EPR effect [34]. This may decisively contribute to enhance the therapeutic effect of the nanoformulations in pre-clinical tests, which are not translated in human trials. Another possible limitation is the use of cell lines rather than patient-derived tumor xenografts. Cell lines are prone to suffer changes by the several in vitro passages overtime and may fail to reflect tumour heterogeneity even in xenografts [34,35]. In contrast, several patient-derived tumour xenografts have been validated for drug testing for colorectal [212], hepatocellular [213] and pancreatic [214], tumours and have shown to provide a more accurate approximation to human cancers. Moreover, the use of orthotopic models constitute more promising alternatives than the most commonly used subcutaneous xenografts, since the later frequently metastasize and may be more representative of advanced stages diseases. Taken together, the most promising animal models for targeted drug delivery may be orthotopic xenografts of human tumors, since it reproduces the histology, molecular nature and metastatic patterns of most human tumors at advanced stages [35,215]. Another important issue that was not fully evaluated in any of the studies was the ability of the guided nanotherapeutics to deliver drugs to less vascularized areas within the tumours. We believe this will be in the future one of the major challenges posed to guided nanotherapeutics and anticancer therapies in general.

Altogether, the past twenty years have been dedicated to establishing the molecular background for the development of efficient target drug delivery strategies. We envisage that the future of digestive tract cancer management may bring low-toxicity multi-target nanodrugs endowed with capability to diffuse to non-vascularized tumour areas. These multivalent constructs may include both ligands for the vasculature and tumour cells, namely through ligands for the bulk of the tumour as well as tailored solutions against more aggressive clones, meaning overcome many of the mechanisms conferring drug resistance and side effects on the frail patients.

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Conflict of interests

The authors declare no conflict of interests

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Table 1. Preclinical studies for liver tumours involving targeted NP loaded with chemotherapeutic agents.

Ligand- class	Receptor	Drug-NP Platform	Animal models ¹	Outcome	Side-effects	Ref
				Compared to free drug and/or non targeted NPs		
Carbohydrate/ polysaccharide						
Galactosamine	ASGPR	Mitomycin C-Xyloglucan	Cell line HepG 2 xenograft	14-fold increase in drug uptake; 15-fold increase in vivo tumoral accumulation; 45% decrease in tumor volume; 27-fold increase in life span.	Not evaluated	[38]
Galactosamine	ASGPR	DOX-Xyloglucan	Cell line HepG 2 xenograft	Increased drug uptake; 45% decrease in tumor volume; Increased tumor growth inhibition.	Not evaluated	[47]
Galactose	ASGPR	5-FU-Chitosan	Cell line H22 xenograft	Increased tumoral drug accumulation; 75% decrease in tumour size.	Reduced toxicity	[64]
Galactose	ASGPR	5-FU-Chitosan	Cell line H22 xenograft (Orthotopic)	1.5-fold increase in tumoral drug accumulation; Decrease in tumour size.	Undetectable	[49]
Galactose	ASGPR	5-FU-Chitosan	Cell line H22 xenograft (Orthotopic)	85-fold increase in tumoral drug accumulation; Decreased tumour size; Increased life span; Increased cytotoxicity in vitro; Increased tumor cell apoptosis induction	Undetectable	[59]
Lactose	ASGPR	DOX-Liposome	Cell line HepG 2 xenograft	4-fold increase in drug uptake; 8-fold increase in tumoral drug accumulation; 81% decrease in tumour growth; Increased life span.	Not evaluated	[58]
Protein/Peptide						
T7	Transferrin receptor	DOX-dendrimer	Cell line Bel-7402 xenograft	Increased drug uptake; 164-195% decrease in tumour volume.	Undetectable	[73]

A54	ND	DOX- iron oxide	Cell line BEL-7402 xenograft	Increased cytostatic effect; Tumoral interstitial accumulation; Absence of anti tumor effect <i>in vivo</i> .	Not evaluated	[71]
A54	ND	DOX- chitosan	Cell line BEL-7402 xenograft	Increased drug uptake; Internalization capability; 3.4-fold increase in tumoral drug accumulation; Increased tumor growth inhibition <i>in vivo</i> .	Reduced toxicity	[37]
RGD	integrins $\alpha v \beta 3$	PTX- PLGA	Liver tumor xenograft (Orthotopic)	Increased drug uptake <i>in vitro</i> ; Increased tumor growth inhibition; Increased life span.	Undetectable	[69]
Apolipoprotein A1	class B type 1 (SR-B1) receptor	DOX-HDL	Cell line LCI-D20 xenograft (Orthotopic)	Increased drug uptake; Internalization capability; Increased tumoral drug accumulation; 38% decrease in tumor size; Increased tumor growth inhibition.	Loss of body weight	[87]

MoAb/Fab

CD 44	CD 44 ⁺ cells	DOX-Liposome	Cell line HepG2 xenograft	7-fold increase in tumoral drug accumulation; Increased tumour growth inhibition; Increased life span; Increased tumor cell apoptosis induction.	Reduced toxicity	[88]
Anti- VEGF	VEGF receptor 2	DOX-Liposome	Cell line BNL xenograft	Increase drug uptake; Internalization capability; Increased tumour growth inhibition; Accumulation in the walls of blood vessels	Not evaluated	[89]
HAb 18	ND	DOX- PEG- PLGA	Cell line HepG2 xenograft	Specific binding leads to 1.5 fold increase in drug uptake; 64% decrease in tumor growth.	Undetectable	[90]

Vitamins

Folate	Folate receptor	DOX- SPION	Cell line Vx2 injection into rabbits and DEN injection into rats	19-fold decrease in tumor volume from rats; 21.5-fold decrease in tumor volume from rabbits; Increased tumor growth inhibition; 23% decrease in mortality; Tumor cell apoptosis induction and proliferation inhibition.	Reduced toxicity	[97]
Others molecules						
Glycyrrhetic acid		ND	DOX-alginate (ALG)NP	Cell line H22 xenograft	Increased drug uptake; 79% increase in tumor growth inhibition; 40% decrease in mortality; Tumor cell apoptosis induction and cytostatic effect;.	Undetectable [10]
Glycyrrhetic acid		ND	DOX - Chitosan	Cell line QGY-7703 xenograft	Increased tumor growth inhibition; Increased cytotoxicity <i>in vitro</i> and <i>in vivo</i> .	Not evaluated- [102]
Glycyrrhetic acid		ND	PTX Chitosan	Cell line H22 xenograft	88% increase in tumor growth inhibition.	Undetectable [48]
Glycyrrhetic acid		ND	Docetaxel-Liposome	Cell line SMMC-7721 xenograft	Increased drug uptake; Internalization capability; Tumor growth inhibition <i>in vitro</i> and <i>in vivo</i> .	Not evaluated [103]
Hyaluronic acid		ND	PTX-liposome	Cell line HepG2 xenograft	Increased pH-mediated drug uptake; Increased tumoral drug accumulation; Increased tumor growth inhibition; Enhanced cytotoxicity <i>in vivo</i> and <i>in vitro</i> .	Not evaluated [60]
Hematoporphyrin	LDL receptor	DOX - Albumin	Cell line HepG2	Increased drug uptake; 33-fold increase in tumoral drug; 4-fold decrease in tumor size.	Undetectable	[62]

1-aminolactose	ND	DOX - liposome	Cell line AH 66 xenograft	Increased drug uptake; Increased tumor growth inhibition.	Not evaluated	[63]

¹Unless otherwise stated the cell lines were subcutaneously engrafted into athymic mice.

¹Unless otherwise stated the cell lines were subcutaneously engrafted into athymic mice.

ND: Not Described

Table 2. Preclinical studies for colorectal tumours involving targeted NP loaded with chemotherapeutic agents.

Ligand	Receptor	Drug-NP Platform	Animal model ¹	Outcome		Ref
				Compared to free drug and/or non targeted NPs	Side-effects	
Carbohydrate						
Galactosamine	ASGPR	DOX - DGLGA	Cell line CT-26 NL-17 xenograft	Absence of therapeutic effect; Enhanced cytotoxicity <i>in vitro</i> and <i>in vivo</i> ; Tumor growth was not suppressed by any formulation	Not evaluated	[108]
Peptide						
APRPG	ND	DOX PEG-Liposome	Cell line C26 NL-17 xenograft	Complete tumor growth inhibition; Increased life span.	Reduced toxicity	[116]
APRPG	ND	DOX - Liposome	Cell line C26 NL-17 xenograft	Increased tumor growth inhibition in Dox-resistant tumors; Tumor cell apoptosis induction.	Not evaluated	[113]

APRPG	ND	CNDAC-Liposome	Cell line C26 NL-17 xenograft	Increased tumoral drug accumulation; Increased tumor growth inhibition; Increased life span.	Undetectable	[115]
K237	Kinase insert domain receptor	PTX- PEG- PLA	Cell line HCT-15 xenograft	Increased tumoral drug accumulation; 8-fold decrease in drug therapeutic dosage; Increased tumor growth inhibition.	Not evaluated	[118]
Cyclic RGD	$\alpha\beta 3$ integrins	DOX - Liposome	Cell line C26 NL-17 xenograft	Increased drug uptake; Internalization capability; Increased tumoral drug accumulation; Increased tumor growth inhibition.	Not evaluated	[111]
MoAb/ Fab'						
Anti-VEGFR	VEGF receptor 2	DOX - PEG-Liposome	Cell line HT-29 xenograft	Decreased tumor volumes.	Undetectable	[112]
AR- 3	ND	5-FU- Liposome	Cell line HT-29 xenograft	Decreased tumor mass to 5%; Enhanced cytotoxicity.	Not evaluated	[130]
GAH	GAH	MCC465-Liposome	Cell lines WiDr-Tc and SW837 xenografts	70-80% decrease in tumor size.	Not evaluated	[169]
Lipid						
TRX-20	Chondroitin sulfate	Cisplatin- PEG-Liposome	Cell line HT-29 xenograft	Increased tumor growth inhibition and metastasis suppression.	Not evaluated	[135]
Aptamer						

STR1	MUC-1	EPI- SPION	Cell line C26 xenograft	Increased drug uptake; Internalization capability; Increased tumoral drug accumulation; 3-fold increase in tumor growth inhibition.	Reduced toxicity	[138]

¹Unless otherwise stated the cell lines were subcutaneously engrafted into athymic mice.

ND: Not Described

Table 3. Preclinical studies for gastric tumours involving targeted NP loaded with chemotherapeutic agents.

Ligand	receptor	Drug-NP Platform	Animal model ¹	Outcome	Side-effects	Ref
				Compared to free drug and/or non targeted NPs		
				Protein/Peptide		
Transferrin	Transferrin and EGFR receptor	Cisplatin-PEG-Liposome	Cell line MKN45P xenograft	Increased tumoral drug accumulation; Internalization capability; Increased life span.	Not evaluated	[145]
KLP	$\alpha 3\beta 1$ integrins	DOX-Liposome	Cell line AZ-P7a xenograft	Increased tumoral drug accumulation; 64-fold increase in tumor growth inhibition.	Undetectable	[151]
RGD	$\alpha v\beta 3$ integrins	DOX and Octreotide-Liposome	Cell line AGS xenograft	Increased tumor growth inhibition <i>in vivo</i> and <i>in vitro</i> .	Reduced toxicity	[152]
				MoAb/Fab ¹		
N-12A5	Erb-2	DOX-PEG-Liposome	Cell line N-87 xenograft	Increased tumoral drug accumulation; 16- fold increasing in binding;	Not evaluated	[164]
Anti CEA	CEA	DOX-Liposome	Cell line MKN-45 xenograft	18 % decrease in tumor weight; Increased tumor growth inhibition.	Not evaluated	[167]

GAH	GAH ⁺ cells	MCC465-Liposome	Cell line B37 xenograft	70-80% decrease in tumor size.	Not evaluated	[169]
GAH	GAH ⁺ cells	DOX-Liposome	Cell line MKN45 xenograft	Increased tumor growth inhibition in free drug resistant tumorsGAH binding and Dox was efficiently delivered into cell nuclei.	Undetectable	[202].
GAH	GAH ⁺ cells	MCC-465 and DOX-Liposome	Cell line MKN45 xenograft	Increased drug uptake; Internalization capability; Increased tumor growth inhibition in free drug resistant tumors.	Not evaluated	[134]

¹Unless otherwise stated the cell lines were subcutaneously engrafted into athymic mice.

ND: Not Described

Table 4. Preclinical studies for pancreatic tumours involving targeted NP loaded with chemotherapeutic agents.

Ligand	Receptor	Drug-NP Platform	Animal model	Outcome		Ref
				Compared to free drug and/or non targeted NPs	Side-effects	
				Fab´		
Transfer rin	Transferrin receptor	Gemcitabine - liposome	Cell line Panc02 xenograft	Specific uptake; Increased life span; extended survival median; Increased tumor cell apoptosis induction.	Not evaluated	[148]
Anti CD 44v6	CD 44v6 receptor	AS- PEG-PDLLA	Cell line PANC-1 xenograft	Increased tumoral drug accumulation; 81% decrease in tumor volume; Increased tumor growth inhibition; Increase apoptosis.	Reduced toxicity	[190]
				Peptide		
cRGD	αvβ3 integrins	Abraxane or gemcitabine - lipid nanogel	Cell line R40P xenograft (Orthotopic)	15-fold increase in tumor growth inhibition; Increased metastasis suppression; Increased tumor cell apoptosis induction.	Not evaluated	[175]

¹Unless otherwise stated the cell lines were subcutaneously engrafted into athymic mice.

ND: Not Describe

NEW TRENDS IN GUIDED NANOTHERAPIES FOR DIGESTIVE CANCERS: A BRIEF SYSTEMATIC FOUR-YEAR UPDATE (2015-2019) FOR GASTRIC CANCER

Gastric cancer remains a critical health burden and continues ranked amongst the top ten tumours in terms of incidence and mortality [1]. Disease management is frequently challenged by the lack of effective and less toxic alternatives to surgery and radio and/or chemotherapy, with half of patients developing metastasis within 5 years after treatment [2]. Moreover, many patients present a fragile health status, being unable to tolerate or complete systemic treatment due severe side-effects [3]. This urges the introduction of more precise and safer therapeutics capable of overcoming chemoresistance and serving a wide range of patient subgroups.

Nanoencapsulation of chemotherapeutic agents within biocompatible polymeric or lipid matrices holds great potential to improve the pharmacokinetics and efficacy of conventional chemotherapy while reducing systemic toxicity [4-6]. According to our previous systematic review [7], engineering the nanoparticles (NPs) surfaces with specific ligands for cancer cells, namely monoclonal antibodies or antibody fragments and other types of molecules (small peptides, proteins, metabolites) provides the necessary means to target more aggressive clones, improving the selectivity and efficacy of nanodelivery vehicles[8]. This systematic review focused on preclinical studies *in vivo* and clinical trials for liver, colorectal, gastric and pancreatic cancers published until 2015. Until 2015 there were 47 studies with potential to translate into clinical applications in a near future. All consensually supported the higher efficacy and safety of drug nanoencapsulation and targeting in comparison to non-targeted molecular constructs and free anti-cancer agents. Amongst the most promising classes of ligands were peptides/small proteins and antibodies/antibody fragments, with affinity

to tumor vasculature and cancer cells. Notably, 8 of the studies focused on gastric cancer.

Envisaging a comprehensive reassessment of the state-of-the-art, we have applied the query for the period between 2015 and 2019 (Query: "Nanoparticles"[9] OR "Liposomes"[All Fields] OR "Polymeric"[All Fields] OR "Inorganic"[All Fields] OR "Dendrimers"[All Fields] OR "Mesoporous silica"[All Fields]) AND ("Active"[All Fields] OR "Targeted"[All Fields] OR "Tumor targeting"[All Fields] OR "multifunctionalized"[All Fields] OR "Ligand"[All Fields] OR "Antibody"[All Fields] OR "Antibody Fragments "[All Fields] OR "Aptamers"[All Fields] OR "Glycoproteins"[All Fields] OR "Peptides"[All Fields] OR "Vitamins"[All Fields]) AND ("Antineoplastic Agents"[9] OR "Doxorubicin"[All Fields] OR "Vincristine"[All Fields] OR "Paclitaxel"[All Fields] OR "Cisplatin"[All Fields] OR "methotrexate"[All Fields] OR "camptothecin"[All Fields]) AND ("drug delivery"[All Fields] OR "therapeutic use"[All Fields] OR "drug carriers"[All Fields] OR "drug effects"[All Fields]) AND (("stomach"[MeSH Terms] OR "stomach"[All Fields] OR "gastric"[All Fields]) OR colorectal[All Fields] OR ("colon"[MeSH Terms] OR "colon"[All Fields]) OR "rectal"[All Fields] OR ("pancreas"[MeSH Terms] OR "pancreas"[All Fields]) OR ("liver"[MeSH Terms] OR "liver"[All Fields]) OR hepatic[All Fields]) OR ("oesophagus"[All Fields] OR "esophagus"[MeSH Terms] OR "esophagus"[All Fields])). The number of identified studies more than doubled in relation to the previous review, denoting tremendous advances in the field (Figure 1). However, the pool of novel reports for gastric cancer remained very similar. Moreover, only one of the previously identified studies, concerning the exploitation of anti-human MAb GAH to target drug loaded polyethyleneglycol (PEG)-modified liposomes to gastric cancer cells, progressed to phase II clinical trials [10]; however, no results have been published yet. As such, this study was not included in Table 1. Concerning the new reports (described in detail in Table 1), most explored different types of biocompatible nanoconstructs (PLGA; chitosan; mesoporous silica) with different types of loads (5-FU; irotecan; doxorubicin) functionalized with hyaluronic acid to target CD44 overexpressed by cancer cells. While elegant, the efficacy

of this targeting strategy maybe challenged in the future by the overexpression of CD44 also by healthy cells in many human organs. Another relevant study exploited folic acid to target folic receptors, overexpressed on most cancer tissues and showing limited expression in healthy tissues and organs [11]. This strategy has also been widely exploited in the past, with demonstrated therapeutic efficacy in preclinical settings for gastrointestinal cancer, warranting now clinical translation. In different settings, AHNP and hyaluronic acid fragments were used synergistically to improve SN38-PLGA loaded targeting to cancer cells. Interestingly, this was the only study exploiting multi-valent targeting; nevertheless, its upfront positive results highlight the importance of multiple targeting to tackle significant tumour molecular heterogeneity. In addition, Yamamoto *et al.* has demonstrated the potential an anti-Tissue factor (TF) F(ab')₂ antibody fragment against cancer cells. Tissue factor is a transmembrane glycoprotein that localizes the coagulation serine protease factor VII/VIIa (FVII/VIIa) to the cell surface [12]. This glycoprotein is significantly overexpressed by cancer cells and plays key functional roles in cancer, including metastasis, tumor growth, and tumor angiogenesis [13]. Finally, used the therapeutic antibody transtuzumab as a vector for guiding 5-FU-Polycaprolactone (PCL) to HER2 cancer cells. Notably, the superior therapeutic efficacy and safety provided by all these approaches in comparison to non-targeted NPs and the free drugs was confirmed *in vitro* and *in vivo*, reinforcing our previous conclusions [14].

In summary, collectively the two systematic reviews demonstrate the benefit of targeting NPs for therapeutic efficacy and safety. They also show that the field has been very active, specially over the past four years, exploiting different targets and molecular nanoconstructs. However, no solutions have yet reached clinical translation. Moreover, most of the targeted molecules remain challenged by low tumour specificity, re-centering the spotlight in biomarker discovery. In fact, the molecular engineering know-how is currently sufficiently mature to support effective targeted therapies for cancer, depending on a careful election of the targeted receptor. However, this aspect remains challenged by significant

intra- and inter-tumoural molecular heterogeneity. According to chapter I, targeting altered glycosylation such as the STn and SLe^A antigens holds great potential towards this end. Surprisingly, the exploitation of cancer-associated glycans has not yet been attempted in the context of drug delivery using targeted nanovehicles. Nevertheless, the necessary rationale for novel and improved therapeutics against gastric cancer has been created and great advances are expected soon, pushed by outstanding developments in the cancer biomarker discovery field.

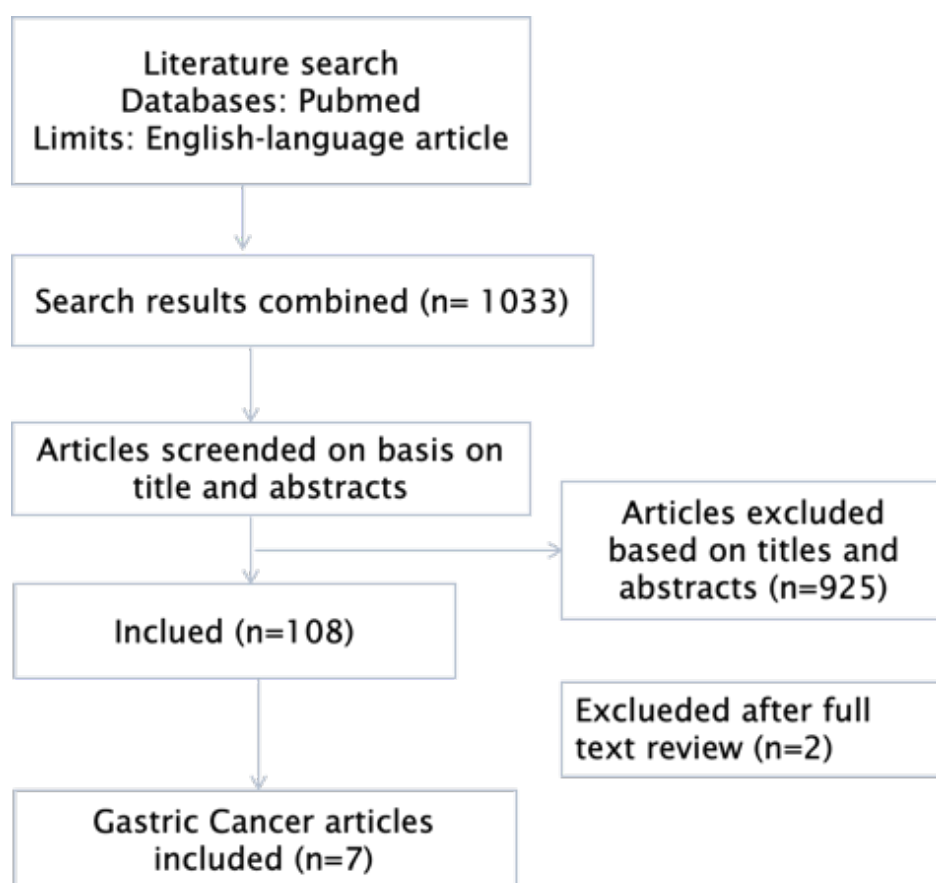


Figure 1. Flowdiagram showing the different phases of the systematic review

Table 1. Preclinical studies for gastric tumours involving targeted NP loaded with chemotherapeutic agents.

	Receptor	Drug-NP Platform	Animal model ¹	Outcome	Side-effects	Ref
				Compared to free drug and/or non-targeted NPs		
MoAb/Fab ¹						
Anti TF F(ab') ₂	Tissue Factor	Epirubicin-Polymeric Micelles	44As3-xenograft	Functionalized NPs was internalized faster and more efficiently into cytoplasm than non-targeted NPS. <i>In vivo</i> , the therapeutic effect was significantly greater compared with non-targeted NP and free Epirubicin.	No severe body weight loss or toxicity-related death was observed in the NPs groups	[15]
Transtuzumab	HER2	5-FU-PCL	NUGC4-xenograft	<i>In vivo</i> , 5-Fu-TNPs significantly reduced tumor growth, and its antitumor effect was much higher than the free drug.	-	[14]
Protein/Peptide						
AHNP and Hyaluronic Acid	HER2 and CD44	SN38-PLGA	HGC27-xenograft	The synergistic effect of the HA and AHNP specific targeting ligands enhance the cellular internalization of SN38-loaded AHNP/HA-hybrid NPs; the suppression of CD44 and Her2 expression by HA and AHNP moieties also inhibit the growth activity of HGC27 cells. The average tumor volume of mice treated with SN38-loaded AHNP/HA-hybrid NPs was 58.5 and 43.3 folds smaller than uncoated and free drug, respectively.	Mice treated with targeted and non-targeted nanoparticles did not present significant changes in body weight, suggesting lack of toxicity	[16]
Other molecules						

Hyaluronic Acid	CD44	5-FU PLGA	NCI-N87- xenograft	HA coated NPs revealed higher targeting ability; In vivo experiments revealed that HA-NPs significantly decreased tumor growth	-	[17]
Hyaluronic Acid	CD44	Irotecan and 5-FU chitosan	MGC803 - xenograft	HA-NPs exhibited better tumor inhibition ability than non-modified NPS	NPs had relatively lower accumulation in heart and kidney	[18]
Hyaluronic Acid	CD44	Doxorubicin- Mesoporous silica	SGC7901/ ADR- xenograft	<i>In vivo</i> anticancer assays on the SGC7901/ADR tumor-bearing mice model revealed a 44% tumor volume decreased compared with nontargeted and free drug	-	[19]
Folic Acid	Folic Acid receptor	Doxorubicin-Au- BSA	MGC803 - xenograft	Compared to free DOX, Au-BSA-DOX-FA showed no pathological changes, a more profound inhibition of cancer cell growth, a more significant decrease in tumor volumes	No severe body weight loss when compared with free drug	[11]

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AIMS & STUDY OUTLINE

Aims & Study Outline

GC is the third leading cause of cancer-related death worldwide [1] and its management is challenged by the lack of efficient molecular tools to assist clinical decision and effectively guide targeted therapeutics [2]. Particularly, patient stratification has been mostly based on clinicopathological features that fail to reflect the complex molecular heterogeneity presented by gastric tumours [3]. Moreover, the response to conventional therapeutic options (surgery, chemo/radiotherapy) has been insufficient to avoid disease progression and poor outcome [4]. Moreover, it is many times accompanied by severe co-morbidities and toxicity, limiting its potential benefit and generalization to fragile and elderly patients [5]. These aspects urge the translation of intense omics studies conducted over the past five years, and mostly of genomics and transcriptomics nature [6-8], into clinically relevant applications to aid precision oncology. Moreover, it sets the focus on the identification of targetable biomarkers of cancer-specific nature that may lead to more effective therapeutics showing less off-target effects.

According to Section I, a reinvestigation of classical glycan biomarkers SLe^A and STn, that have served us well for non-invasive GC detection and patients follow-up after surgery, may provide the necessary means towards improved GC management. The current state-of-the-art (**Section I**) supports the decisive functional role of these glycans in many aspects of GC progression and dissemination, including activation of relevant oncogenic pathways, chemoresistance, migration, invasion, metastasis development and immune escape [9, 10]. These aspects associated with their cell-surface nature and low or absent expression in most healthy suggests potential for developing targeted therapeutics. Nevertheless, the clinical context underlying the expression of these glycans in GC remains poorly understood. In fact, it has never been comprehensively addressed in a large patient series including all stages of the disease. Moreover, their inclusion in models exploiting other biomarkers commonly used in the clinics (*e.g.* E-cadherin and Ki-67) has not yet been performed. Finally, the nature of the

glycoproteins carrying these glycoepitopes remain mostly unknown. This aspect has significantly delayed a more profound understanding of glycans functional roles in cancer and ultimately the identification of cancer-specific signatures for precise therapeutic intervention. On a different perspective, **Introduction Section** highlighted that the encapsulation of therapeutic drugs in biocompatible nanovehicles constitute a tremendous opportunity to improve the efficacy of conventional chemotherapy, while significantly lowering its side-effects. It was also concluded that engineering molecular nanoconstructs with antibodies to selectively target more aggressive clones improves its therapeutic efficacy. Nevertheless, the exploitation of glycans towards this end has never been attempted in GC, providing new avenues for intervention.

Considering these observations and facing current GC management limitations, this thesis has established the following main objective: Define the clinical significance of altered glycosylation in GC and set the molecular foundations for glycan-based nanotherapeutics envisaging precision oncology. Accordingly, the following specific aims were established: i) provide a clinical context for STn and SLe^A expressions in GC, envisaging molecular models for patients stratification; ii) set the molecular foundations for glycan-targeted nanovehicles for delivering chemotherapy agents to more aggressive cancer cells; iii) Glycoproteomics characterization of GC envisaging unique molecular fingerprints for precise cancer targeting.

As general strategy, the results sections starts by addressing the clinical significance of STn and SLe^A in GC, alone and in combination with other classical gastric cancer biomarkers explored in clinical settings (**Chapter 1**). Envisaging this objective, it elected to include E-cadherin, used to define epithelial phenotypes generally facing better prognosis, and frequently lost in more aggressive mesenchymal-like tumours [11] as recently highlighted by comprehensive multi-omics and clinical studies [12, 13]. The biomarker panel also included an evaluation of the tumour proliferation index based on the expression of nuclear protein Ki-67 (**Chapter 1**). High Ki-67 has been associated with uncontrolled tumour

proliferation and, consequently, decreased prognosis [14]; however, a reduction in tumour proliferation may occur for subgroups of tumours enriched for quiescent and more aggressive stem-cell phenotypes [15]. Their evaluation is routine in most pathology laboratories and maybe easily incorporated in broader molecular panels that include glycosylation. As such, this chapter attempts to bring together STn, SLe^A, E-cadherin and Ki-67 to create a novel comprehensive patient stratification models of clinical utility. The establishment of such models is expected to generate decisive insights on the clinical significance of STn and SLe^A in GC. Furthermore, it will bring key information to help guiding downstream targeted glycoproteomics studies towards cancer-specific glycoprotein signatures to target cancer cells (**Chapter 3**). **Chapter 2** builds on the findings from **Chapter 1** to develop a biocompatible glycoengineered nanoparticle capable of delivering chemotherapeutic agents to more aggressive GC cells. For proof-of-concept this study elected an anti-proliferative agent (5-fluorouracil) and paclitaxel that is more effective against cells in different phases of the cell cycle. Notably, these agents are frequently part of therapeutic regimens adopted for GC [16, 17]. Nanoscale drug delivery vehicles are expected to improve the efficacy of chemotherapeutic agents, while reducing their cytotoxicity. Therefore, this may constitute an important milestone towards glycan-based targeted therapeutics against GC. Given the pancarcinomic nature of these glycans, the translation of these solutions to other models may also be envisaged. Finally, **Chapter 3** builds on the molecular rationale generated in **Chapter 1** to propose a targeted glycoproteomics approach that may lead to the precise identification of cancer-specific glycosignatures. it explores a representative panel of GC cell models reflecting different clinicopathological natures of the disease envisaging this end. Briefly, it brings together different types of lectin-affinity chromatography strategies to selectively isolate glycoproteins expressing the glycosignatures of interest. The isolated glycoproteins were subsequently identified by high-resolution mass spectrometry after enzymatic digestion. Bioinformatics tools combining different omics

(genomics, transcriptomics, proteomics) were then used to comprehensively interrogate the identified glycoproteins for clinically relevant signatures. Potentially promising glycobiomarkers were further validated in clinical samples. This proof-of-concept is expected to set important milestone towards the discovery of clinically meaningful and cancer-specific glycoproteomics signatures, thus fulfilling one of the outstanding challenges identified in **Introduction Section** related with precise cancer targeting.

Altogether, this workflow was designed to provide a molecular framework for clinical intervention in GC at different levels (patient stratification and prognosis, targeted therapeutics) having glycans as starting point. This is anticipated to contribute to improve the management of particularly aggressive tumours, thus improving quality of life and survival.

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CHAPTER 1

**CLINICAL RELEVANCE OF CLASSICAL GLYCOBIOMARKERS IN
GASTRIC CANCER TISSUE SECTIONS**

The information provided in this chapter presents part of unpublished data of a manuscript in preparation by Elisabete Fernandes et al.

This chapter is based in the following unpublished data:

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In preparation.

Clinical Relevance of Classical Glycosylation Biomarkers in Gastric Cancer: STn And sLe^A Overexpression Helps Defining Poor Prognosis And Associates with Cancer Dissemination

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Abstract

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide [1], due to high molecular heterogeneity and the absence of more effective and less toxic targeted therapeutics. Moreover, GC classification based on clinicopathological features alone fails to provide accurate patient's stratification, urging the introduction of molecular-assisted precision oncology. Aberrant glycosylation of membrane proteins presented by more aggressive GC cells and translated by STn and SLe^A overexpression's suggests potential to achieve this goal.

As such, this work proposes a molecular panel composed of clinically relevant glycobiomarkers (STn and SLe^A) and classical epithelial differentiation (E-cadherin) and proliferation (Ki-67) markers for identification of patients' subgroups facing worst prognosis. This panel was able to differentiate poor cancer-specific survival (CSS) groups within broadly used GC classifications (Lauren and Borrmann) and several clinicopathological variables (stage, grade, number of lymph node and distant metastasis). Specifically, aberrantly glycosylated, poor proliferative tumours presenting a significant decrease in E-cadherin have emerged as a particularly aggressive molecular subtype in comparison to more proliferative, E-cadherin overexpressing lesions. This aggressive phenotype has defined poor prognosis patients falling within type II and III Borrmann and intestinal type classifications. Moreover, it stratified worst survival amongst patients with T3, N_≤ 2 and M0 tumours, according with the TNM staging system. In addition, clinical stage II and III patients' displaying this aggressive phenotype also presented decreased CSS. These findings support that abnormal glycosylation may contribute to improve the identification of more aggressive tumours and provides a key rational for molecular targeting both primary lesions and control disease dissemination at different levels.

Overall, these findings set the bases for the introduction of glycans in molecular panels for improved GC management. Future studies should include the construction of a more comprehensive molecular panel,

including targeted glycoproteomics towards the identification of targetable molecular signatures.

Introduction

Gastric cancer (GC) is one of the most frequently diagnosed cancers and the third leading cause of cancer-related death worldwide [2]. The mainstay therapeutic options are based on surgery and chemotherapy, which fail to prevent tumour recurrence and progression. As a result, patients median survival does not exceed 10 months [3], despite the introduction of targeted approaches such as trastuzumab (anti-HER2), ramucirumab (anti-VEGFR2), and cetuximab (anti-EGFR) [4-6]. Moreover, the development of more effective and cancer-specific targeted therapeutics has been challenged by high tumour molecular heterogeneity [7], urging the identification of targetable biomarkers.

Another critical aspect in GC management relates the lack of efficient tools to assist patient stratification, therapeutic selection and prognosis. Currently GC this has been largely based on histological features defined by the Lauren's criteria [8], by which gastric tumours are either classified as intestinal (better prognosis), mixed (intermediate prognosis) and diffuse (worst prognosis) type carcinomas. Alternatively, tumours may be ranked based on their macroscopic appearance based on the Borrmann's classification. Borrmann's type I tumours with best prognosis generally present a polypoid nature, type II a fungating growth, type III an ulcerating extension, and type IV comprehends more aggressive lesions with diffusely infiltrating growth with signet ring cells [9]. Even though informative, these classifications are not sufficiently accurate to provide accurate staging. Nevertheless, tumour showing apparently similar histology frequently present differentiated clinical behaviours, challenging these models and urging the introduction of molecular signatures. The AJCC 8th TNM staging system is also used to stratify tumours according to adjacent layer invasion (T), lymph-node involvement (N) and distant metastasis (M) [10]. However, this classification has many pitfalls associated with operator dependence

that bias clinical decisions. Overall, these widespread classifications fail to address the main current patients' management challenges at different levels, including non-invasive detection of the disease, prognostication, therapy selection, patient's follow-up and targetable biomarkers for the introduction of improved and safer therapeutics. Therefore, the identification of dysregulated pathways and candidate drivers seems to be the next logical step to better understand GC carcinogenesis. Facing this need, we attempt to improve pre-existing classifications using clinically relevant molecular GC biomarkers.

Advanced stage GC is characterized by profound alterations in protein glycosylation [11]. Two of the most studied glycans in GC are the sialyl-Tn (STn) antigen, a short-chain glycan resulting from a premature stop in protein *O*-glycosylation, and the sialyl-Lewis A (SLe^A) antigen, a terminal glycoepitope of carbohydrate chains in glycoproteins and glycolipids. These glycoepitopes are absent or very strictly expressed in healthy tissues, being significantly overexpressed in advanced stage digestive tract tumours where they are frequently associated with poor prognosis [12, 13]. STn and SLe^A antigens are widely used in clinical practice for non-invasive follow-up based on the CA72-4 (for STn) and CA19-9 (for SLe^A) tests; however, they lack the necessary specificity and sensitivity for diagnosis and some controversial results exist regarding their potential for accurate patient stratification [14]. Nevertheless, there is a relevant amount of information supporting their involvement in cell invasion and metastasis development, providing a relevant rationale for intervention [15]. Therefore, their incorporation in more comprehensive multibiomarker panels may significantly contribute to improve clinical decisions.

Several molecular subtyping models steaming from genomics and transcriptomics studies have been proposed for GC [16-18]; however these approaches still require refinement facing clinical translation. Nevertheless, they support the importance of defining the proliferation status of GC tumours. Ki-67 has become one of the most commonly used biomarkers for assessing increased cell proliferation in several types of carcinoma [19-21],

including GC [22]. Ki-67 is only expressed in the G1, S, and G2 stages of the cell cycle, but not in G0 stage; hence, Ki-67 usually is regarded as a valuable biomarker of cell growth and proliferation [23, 24]. Moreover, recent metanalysis suggested that Ki-67 overexpression was associated with poor prognosis in GC patients. Also, Ki-67 positive rates may be associated with age, lymph-node metastasis, tumour size, and TNM staging system in GC patients [25]. In addition, abnormal expression of E-cadherin is a common phenomenon in GC, consensually highlighted as having clinical potential by more recent comprehensive studies [26-28]. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent intercellular adhesion and its low expression has been associated with advanced stages and more aggressive behaviour of several cancers [29-33], including GC [34]. Moreover, loss of E-cadherin is a common inducer of epithelial-to-mesenchymal transition (EMT), which is associated with cancer stem cells and drug resistance in human cancer, including GC [35]. However, despite the clinical relevance of addressing proliferation and the epithelial status of the tumour, these biomarkers have never been comprehensively evaluated together and in combination with altered glycosylation in GC.

Based on these observations, the present work aims to address the clinical significance of STn and SLe^A in GC and their associations with the proliferative and epithelial status of the tumour. We anticipate that this may help improving patient stratification and prognostication, while setting the molecular bases for glycan-based targeted interventions.

Material and Methods

Patient and sampling

The screening of STn, SLe^A, Ki-67 and E-cadherin was performed on 258 formalin-fixed paraffin embedded (FFPE) tissue sections collected corresponding 202 primary tumours and 56 correspondent metastasis from 109 male and 93 female patients with a mean age of 64 years (ranging 34–89 years). All patients underwent gastric surgery in the Portuguese Institute of Oncology-Porto (IPO-Porto, Portugal) between 2004 and 2016. Based on

the Lauren classification [36], intestinal type (n=124), diffuse type (n=50) and mixed type (n=22) tumours were selected. According to the Borrmann classification [9], Borrmann type I (n=9), II (n=76), III (n=42) and IV (n=58) tumours were included. Rendering the TNM staging system [10], T1 (n=12), T2 (n=17), T3 (n=105), T4 (n=64), N0 (n=49), N1 (n=40), N2 (n=32), N3 (n=76), M0 (n=151) and M1 (n=36) stage tumours were appended. Lastly, tumours from patients classified with clinical stage I (n=18), II (n=60), III (n=89) and IV (n=35) were also included [10]. All tumour samples were revised by a pathologist according to Lauren's classification and Borrmann grading criteria. All procedures were performed under the approval of the institutional Ethics Committee after patient's informed consent. All clinicopathological information was obtained from patient's clinical records.

Immunohistochemistry

Three micrometres gastric tumour sections were deparaffinized, rehydrated and incubated for 15min with boiling citrate buffer (Vector) or EDTA pH 9. Tissue sections were then exposed to 3-4% hydrogen peroxide for 5min (Leica) and STn SLe^A, Ki-67 and E-cadherin were detected using the Novolink Max Polymer DS Kit (Leica), according to the manufacturer instructions. Positive and negative controls were tested in parallel, including enzymatic controls with an α -neuraminidase from *Clostridium perfringens* (Sigma-Aldrich, Missouri, USA). Experimental conditions for antigen analysis were summarized on **Table 1**. A broad panel of healthy tissues were also screened for both glycobiomarkers.

Table1- Antibody conditions employed in immunohistochemistry procedures.

Antigen	moAb	Antigen retrieval	Dilution
E-Cadherin	NCL-L-E-Cad (Leica)	EDTA pH=9 30min	1:50
Ki-67	MIB-1 (Agilent)	EDTA pH=9 15min	1:150
SLe ^A	CA19.9-203 (Abcam),	Citrate buffer low pH 15min	1:100
STn	B72.3 + CC49 (Abcam)	Citrate buffer low pH 15min	0.5µg/ml

Statistical analysis

Statistical analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 26.0; IBM, Armonk, NY, USA). Chi-square analysis was used to compare categorical variables. Kaplan–Meyer survival curves were used to correlate antigen expression with Cancer-Specific Survival (CSS). Log-rank statistical test was used for curve comparison. Moreover, the Orange software was used to define cut-off values for low and high antigen expression.

Results and discussion

This work focus on defining a clear clinical context for STn and SLe^A expressions in GC, envisaging their exploitation in precision oncology settings, including novel rationales for targeted therapeutics. It also combines glycans with established biomarkers associated with tumour proliferation (Ki-67 expression) and epithelial character (defined by E-cadherin levels) to propose a comprehensive molecular model for patient stratification.

Screening of molecular biomarkers in Gastric Cancer

The screening of STn, SLe^A, Ki-67 and E-cadherin was performed on 202 FFPE tissue sections and 56 correspondent metastasis. Accordingly, most of the studied tumours and metastasis expressed the addressed molecules (90% and 86% of cases positive for STn, 81% and 71% for SLe^A, 92% and 96% for Ki-67, 88% and 79% for E-cadherin, respectively). STn, SLe^A, and E-

cadherin were predominantly found at the cell surface, while Ki-67 showed positive nuclear expression (**Figure 1A**), not being identified any particular pattern of staining. Envisaging to fully disclose its cancer-associated nature, the STn antigen was also evaluated a series of eight healthy tissues (gallbladder, pancreas, lung, stomach, colon, skin, liver and kidney). Notably, STn had a very restricted distribution in healthy tissues, being circumscribed to parietal and goblet cells of the gastrointestinal and respiratory tracts, being expressed in much less extent in comparison to tumour tissues (**Figure 1B**). In tumour samples, STn is highly observed in the invasion fronts of the tumours, in a median extension of $50\% \pm 25.44$. Moreover in GC, SLe^A is expressed in the cell-surface of tumour cells with higher expression in superficial areas, comprising a median expression of $40\% \pm 28.92$. In healthy tissues SLe^A was mainly found in the cytoplasm of goblet cells, in pancreatic acini and in gallbladder epithelial cells secretion vesicles (**Figure 1C**). These observations support the cancer-associated nature of the studied glycoantigens, irrespectively of their clinicopathological nature. It also emphasises the need to engage in targeted glycoproteomics studies envisaging cancer-specific molecular fingerprints. Altered glycosylation may either reflect subpopulations of cancer cells with distinct genetic backgrounds or denote differentiated responses of the cancer cells glycosylation machinery to distinct tumour microenvironments, as suggested by recent reports [37-39]. Notably, immunoassays demonstrated that low-proliferative tumour areas (low Ki-67), known to harbour more aggressive quiescent tumour cells [40-42], were often enriched for STn and SLe^A expressing cancer cells (**Figure 1E**). Future studies focusing on the molecular nature of cancer cells showing altered glycosylation, including a through genetic characterization is required to fully disclose the nature of the sub-populations. A more profound knowledge about the impact of the tumour microenvironment on the glycome will also be critical to disclose the mechanism underlying altered glycosylation in GC. Nevertheless, such observations provide an important

framework for intervention, suggesting that targeted therapeutics addressing both antigens could be beneficial for GC management.

Ki-67 and E-cadherin are detected in tumour samples and healthy tissues (not shown), giving relevant notions on the epithelial differentiation and proliferative state of cells without having a cancer-specific nature. Accordingly, GC tumours presents a median expression of $40\% \pm 23.76$ and $80\% \pm 27.52$ for Ki-67 and E-cadherin respectively. Notably, a correlation was also observed between Ki-67 and E-cadherin expressions, suggesting that tumour proliferation is often accompanied by an epithelial phenotype associated to better prognosis [42-44]. Contrastingly, cell surface aberrant glycosylation has a strong cancer-associated nature, given its absence or poor representativity in healthy tissues, paving the way for targeting opportunities.

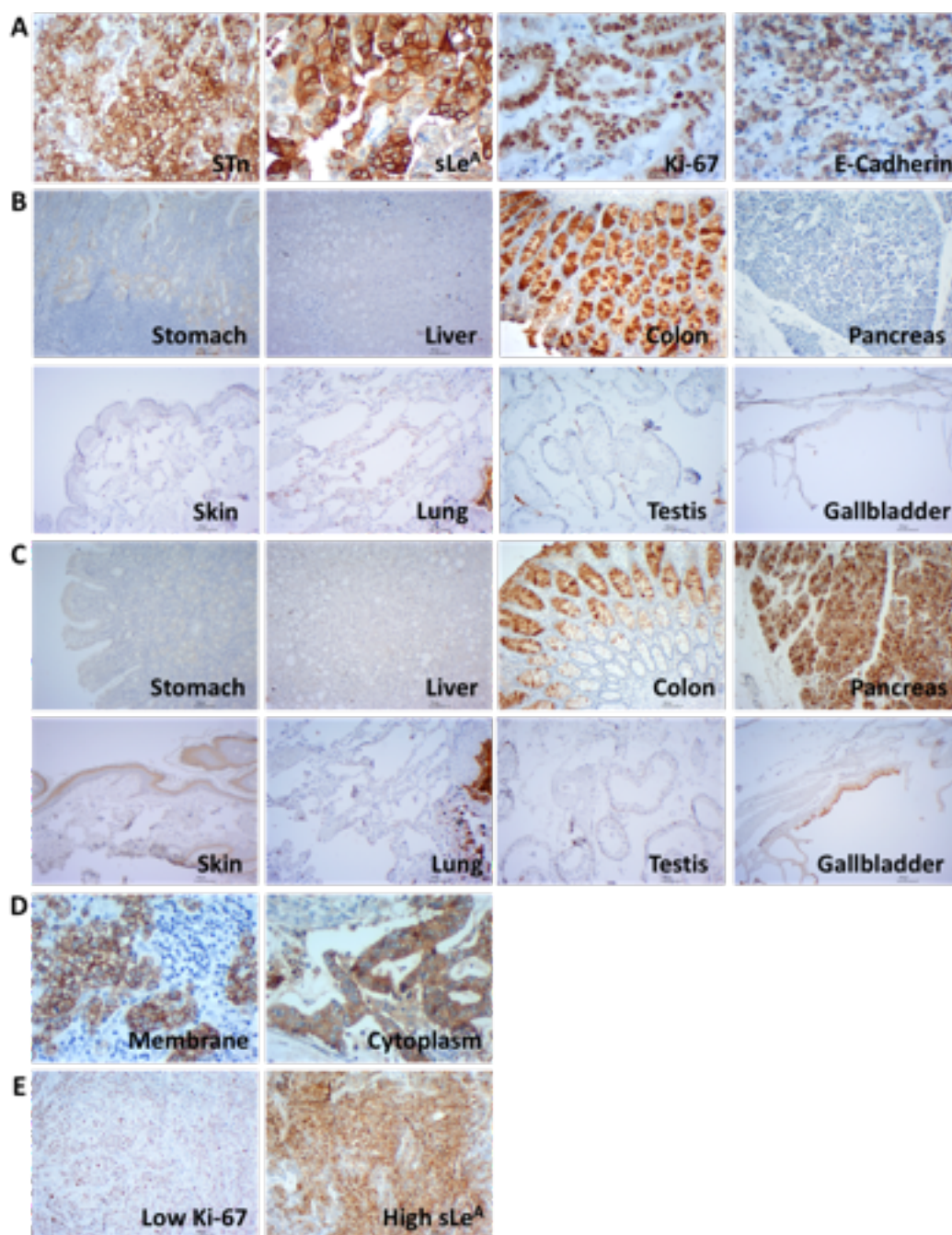


Figure 1. Illustrative expression of (A) STn, SLe^A, Ki-67 and E-cadherin in GC tumour sections. (B) STn, (C) SLe^A and (D) E-cadherin's pattern of expression in tumour tissues. (E) Representative high SLe^A expression in GC tissues co-localizing with low nuclear expression of Ki-67.

Clinical Significance of Altered Glycosylation in Gastric Cancer

For each antigen, a score value was determined by multiplying the staining extension (defined between 0-100% tumour area) by the staining intensity (defined between 0-3). Subsequently, the score was used to define

a threshold for high and low antigen expressions that was latter used to evaluate cancer-specific survival (CSS). The adopted threshold for defining decreased CSS based on STn expression was 120 (arbitrary units), for SLe^A 30, for Ki-67 80 and for E-cadherin 150, below which patients had decreased cancer-specific survival (CSS) (**Figure 2**). When evaluated individually using this criteria, low STn, Ki-67 and E-cadherin identified patients' subgroups with decreased CSS. Contrastingly, sADCv SLe^A expression alone did not contribute to patient stratification regarding CSS (**Figure 2**).

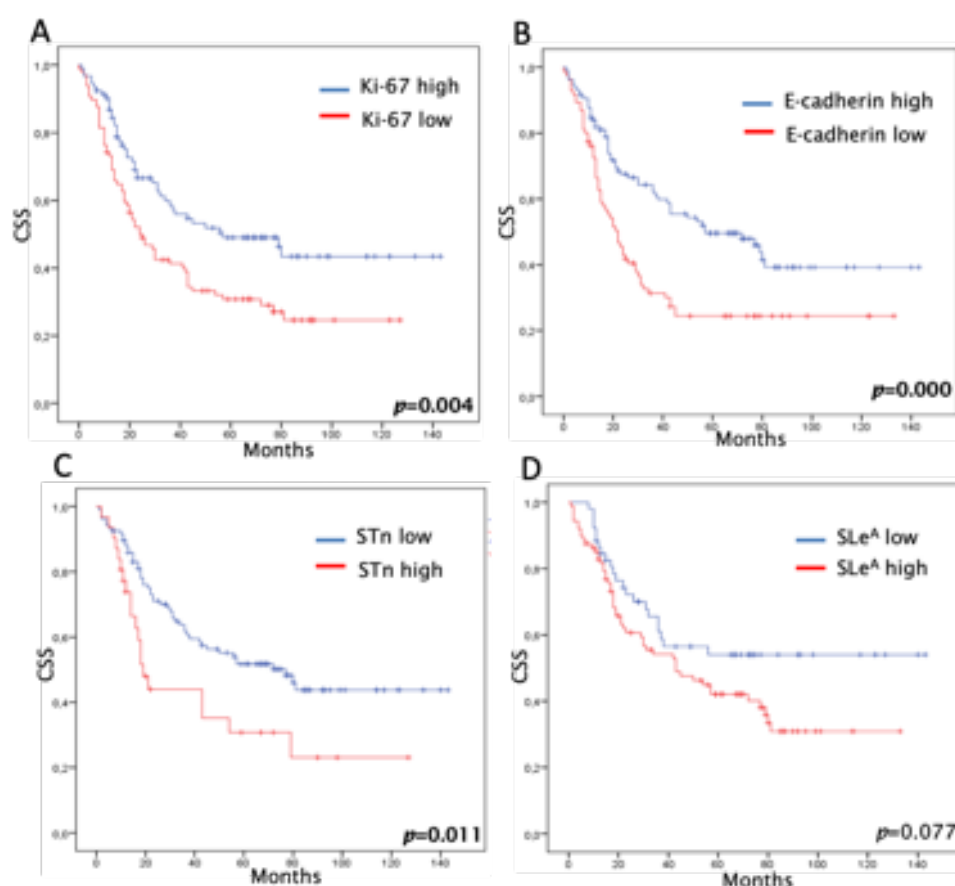


Figure 2. Cancer-specific survival (CSS) of gastric cancer patients according with (A) STn, (B) SLe^A, (C) Ki-67 and (D) E-cadherin expression. Patients exhibiting high STn levels ($p=0.011$) as well as low Ki-67 ($p=0.004$) and low E-cadherin ($p=0.000$) levels display decreased CSS compared to patients with opposite phenotypes. Patients with high SLe^A levels ($p=0.077$) have a trend decrease in CSS.

Furthermore, none of these biomarkers showed statistically significant associations with Lauren's or Borrmann's classifications (**Table 2 and 3**). Moreover, STn and SLe^A levels, as well as Ki-67 status, were not associated

with the stage of the tumour. On the other hand, high E-cadherin levels associated with T1 stage (75% of cases), while low E-cadherin occurred mainly in T3 stage (58.3% of cases) (**Table 4**). These findings reinforce the correlation between loss of E-cadherin and increasingly aggressive tumour status, in accordance with previous reports [34, 45]. In fact, decreased E-cadherin levels have been previously associated with more aggressive tumours, the acquisition of mesenchymal traits, cellular dedifferentiation and metastasis development [44]. In agreement with these observations, low Ki-67 and E-cadherin expressions also correlated with increased lymph node involvement ($N > 1$). In turn, high STn expression correlated with N status above 2, while low expression was associated with the absence of lymph-node involvement (N0). These findings suggest that poorly proliferative tumours with less cohesive phenotypes and overexpressing STn may present higher rates of lymph-node metastasis. Notably, high SLe^A expression did not associate with lymph-node metastasis (**Table 5**); however, it was strongly linked to distant metastasis (80.6% of cases), in clear contrast with all the other evaluated biomarkers (**Table 6**). Interestingly, these results are supported by the biological roles played by STn and SLe^A in cancer dissemination. Namely, STn is frequently observed in tumour invasive fronts and is associated with increased motility, while SLe^A is a known ligand of E-selectin, mediating cancer cells binding to endothelial cells, cancer cells extravasation from tumour sites and hematogenous metastasis [46, 47]. These observations also highlight that STn and SLe^A may be linked to distinct metastasis routes, which warrants future investigation envisaging precise interventions. Finally, regarding clinical stage, low E-cadherin expression correlated with stage III GC (60.7% of cases), while high SLe^A expression correlated with stage IV GC (82.9% of cases) (**Table 7**). In summary, STn and SLe^A overexpression's significantly associated with disease dissemination. Moreover, non-proliferative tumours showing the loss of epithelial features appear to be associated with more aggressive clinicopathological features (increased lymphatic metastasis). Moreover,

expression analysis of above mentioned markers in metastasis reflect their expression in tumour as previously demonstrated by (Table 8) [34, 48].

Given their potential, these biomarkers were then combined envisaging to improve patient stratification and CSS estimation. Two distinct molecular subtypes were identified using this strategy: i) a good prognosis group, comprehending tumours showing marked proliferative and epithelial character (high E-cadherin and high Ki-67) and tumours of hybrid characteristics with either elevated E-cadherin or Ki-67; and ii) a worst prognosis group, composed by tumours of non-proliferative and potentially mesenchymal-like nature, characterized by low-proliferation indexes and the substantial loss of E-cadherin. Nevertheless, future studies must address mesenchymal markers to determine the accuracy of the worst prognosis group classification. Notably, no significant differences were observed for STn and SLe^A levels between both groups, demonstrating that the inclusion of glycosylation had little discriminatory capacity at this level. Nevertheless, elevations in both STn and SLe^A, irrespectively of the E-cadherin and Ki-67 status, could be used to discriminate patients with significantly lower CSS in the better prognosis group (approximately 90 months, $p=0.000$). More importantly, the CSS of these particular tumours showing altered glycosylation (high STn and/or SLe^A) was comparable to the worst prognosis mesenchymal-like subtype. These findings demonstrate that the glycosylation status may help improving tumour classification. Moreover, it highlights that cancer-associated glycans may decisively help driving poorer clinical outcomes, as suggested by their cancer-promoting functional roles. Based on CSS similarities resulting from the combination of proliferative and epithelial features with glycosylation, the groups were then merged into two distinct prognosis subtypes: i) the epithelial and/or proliferative-like subtype, comprehending lesions of clear proliferative and epithelial character but also hybrid tumours showing mild alterations in glycosylation (either STn or SLe^A elevations); ii) the mesenchymal-like subtype, most likely enriched for quiescent stem-cells, comprehending lesions showing marked non-proliferative and non-epithelial features and an heterogenous group of

tumours presenting either elevations in E-cadherin and/or Ki-67 (E-cadherin high/Ki-67 high; E-cadherin high/Ki-67 low; E-cadherin low/Ki-67 high phenotypes) and profound alterations in glycosylation.

This molecular classification was then addressed together with clinicopathological variables aiming towards improved prognostication capacity. We started by addressing tumours ranked according to the Lauren's classification. Notably, our series reflected the survival curves described in the literature [49, 50] whereby the intestinal phenotype presented favourable prognosis (Figure 5A, $p=0.000$), whereas diffuse type tumours had worst prognosis (Figure 5B, $p=0.083$). Mixed type tumours exhibited an intermediate behaviour (Figure 5C, $p=0.103$). Nevertheless, despite clear intestinal histology, tumours showing a mesenchymal-like molecular phenotype presented a significant reduction in survival in comparison to tumours with an epithelial-like character (approximately 20 months, $p=0.000$), improving the discriminatory capacity of this classification. The survival curves presented in Figure 5B also suggested a similar behavior for diffuse type tumours curves facing the proposed molecular stratification, even though showing just a trend towards statistical significance ($p=0.083$). Focus was then set on Borrmann's classification, which presented a decrease in survival with increased in tumor type (I, II, III, IV), as highlighted by Figure 4. The incorporation of our molecular model, also refined the discriminatory capacity of the Borrmann's classification system, enabling the identification of type II and III tumours facing worst prognosis based on the presence of mesenchymal-like molecular features. In addition, we observed that mesenchymal-like phenotypes were discriminative of poor prognosis in stage T3 tumours (**Figure 6**), and for tumours with $N \leq 2$ (**Figure 7**). Moreover, it discriminated poor prognosis subgroups in patients without distant metastasis (M0), highlighting the need for closer follow-ups.

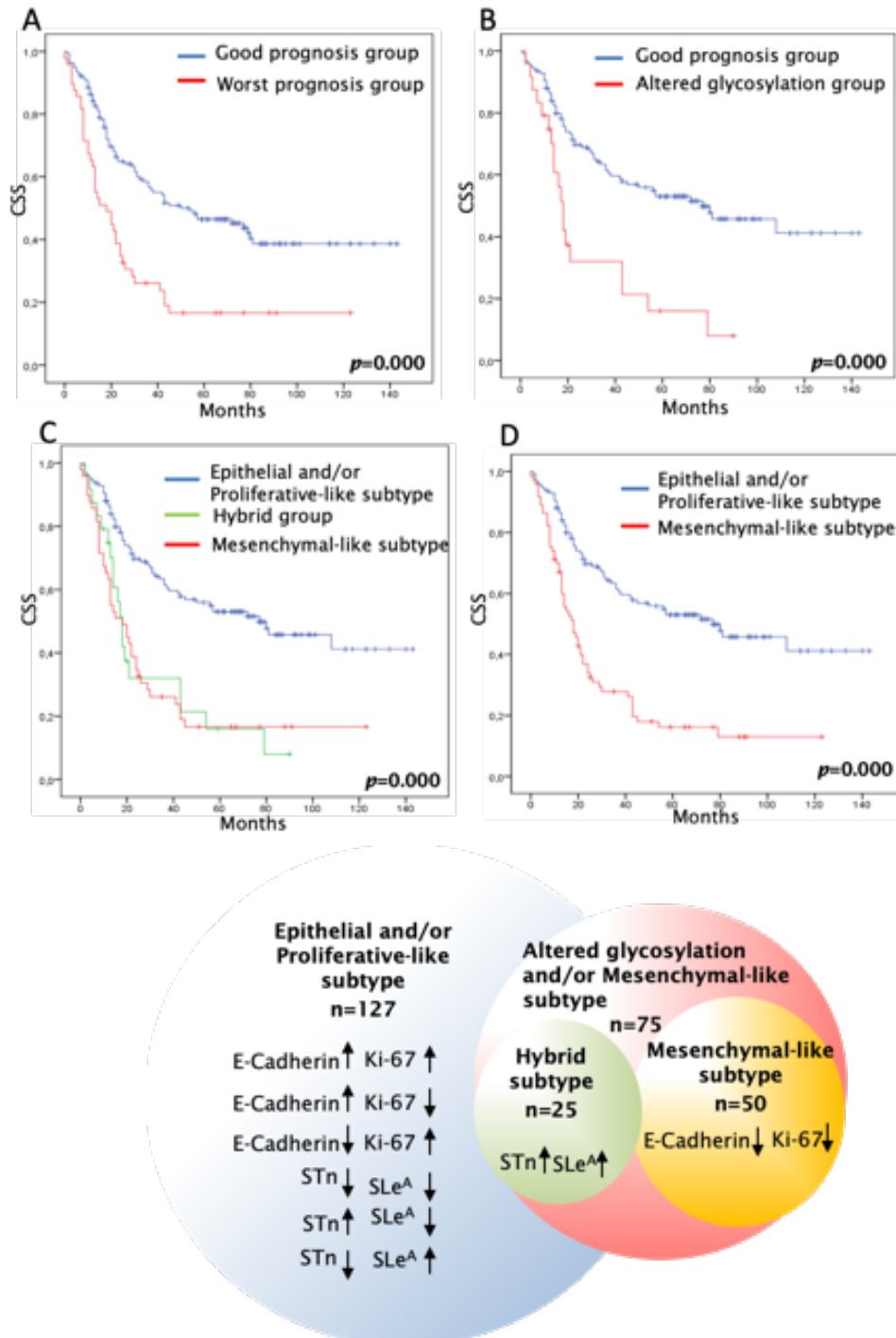


Figure 3. Cancer-specific survival (CSS) of gastric cancer patients according with the expression of the proposed molecular markers SLe^A, STn, Ki-67 and E-cadherin. **(A)** discriminated CSS of group A (low Ki-67 and E-cadherin levels) and group B (low or high Ki-67 and E-cadherin expression), **(B)** discriminated CSS of group B1 (high STn and SLe^A levels) and group B2 (low or high SLe^A and STn expression), **(C)** discriminated CSS of groups A, B and B1; **(D)** discriminated CSS of group C (low Ki-67 and E-cadherin and high SLe^A and STn expressions) and group D (comprising all previous biomarker combinations). Group A displays decreased CSS than group B patients ($p=0.000$). Group B1 have reduced CSS than

group B2 patients ($p=0.000$). Groups A and B1 showed poorer CSS than group B patients ($p=0.000$). Finally, group C patients exhibit diminished CSS than group D ($p=0.000$). (E) schematic representation of patient's subgroups.

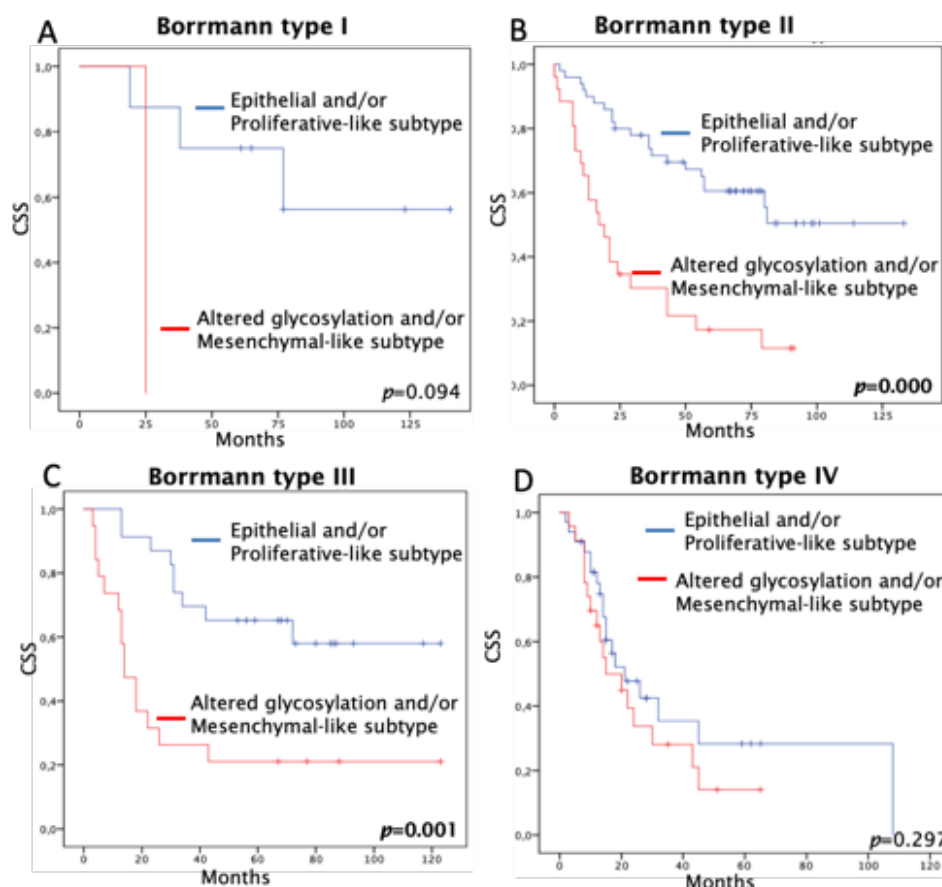


Figure 4. Cancer-specific survival (CSS) of patients composing groups C and D within the Borrmann's classification. Discriminated CSS of groups C and B in (A) type I, (B) type II, (C) type III, and (D) type IV Borrmann tumours. Group C patients only have significantly decreased CSS when diagnosed with type II ($p=0.000$) and III ($p=0.001$) Borrmann tumours.

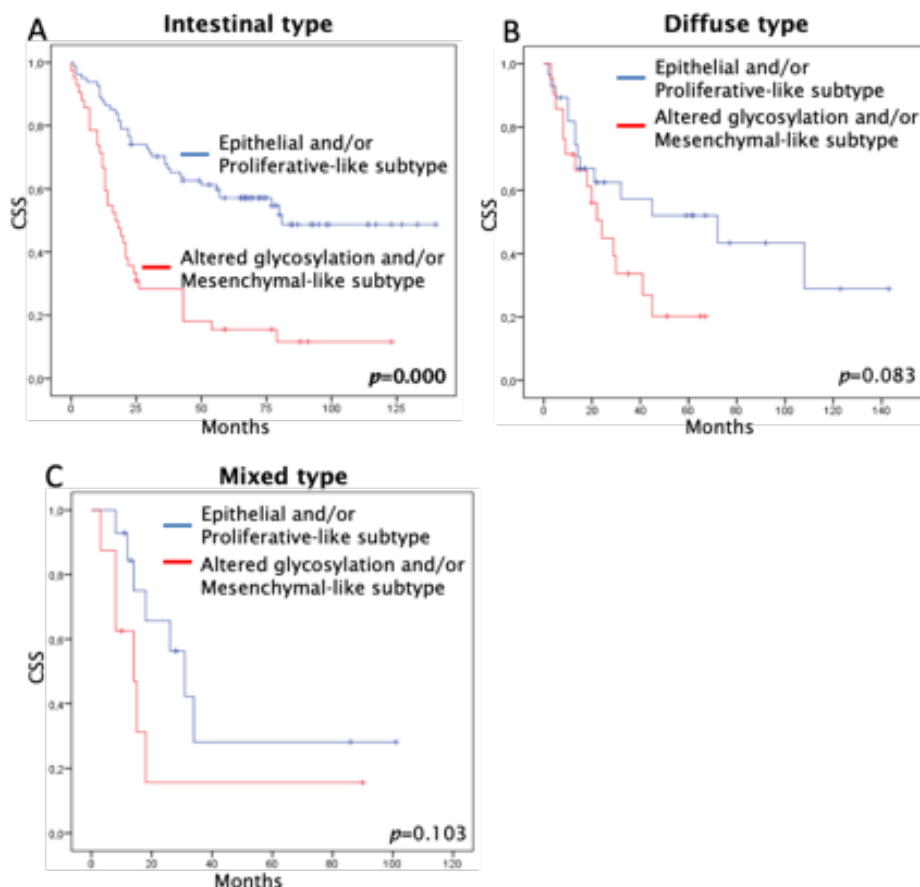


Figure 5. Cancer-specific survival (CSS) of patients composing groups C and D within the Lauren's classification. Discriminated CSS of groups C and B in **(A)** intestinal type tumours, **(B)** diffuse type tumours, and **(C)** mixed type tumours. Group C patients only have significantly decreased CSS when diagnosed with intestinal type GC ($p= 0.000$).

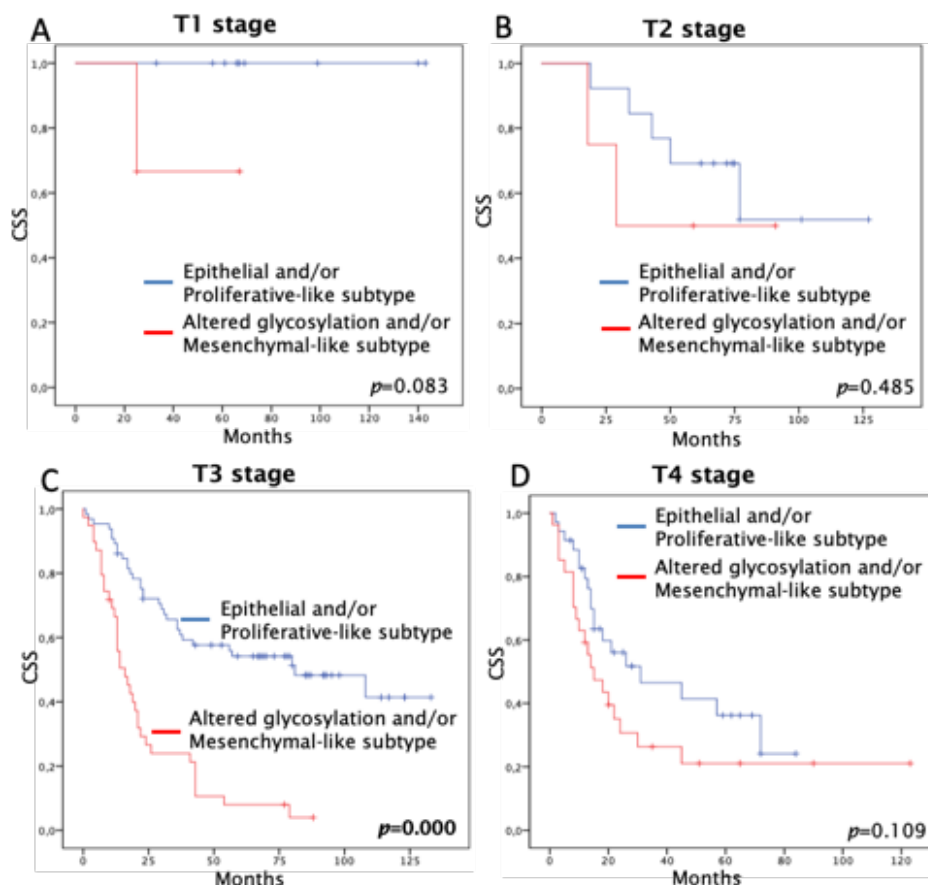


Figure 6. Cancer-specific survival (CSS) of patients composing groups C and D according with the T stage of the TNM staging system. Discriminated CSS of groups C and D in (A) T1, (B) T2, (C) T3 and (D) T4 tumours. Group C patients only have significantly decreased CSS when diagnosed with T3 GC ($p=0.000$).

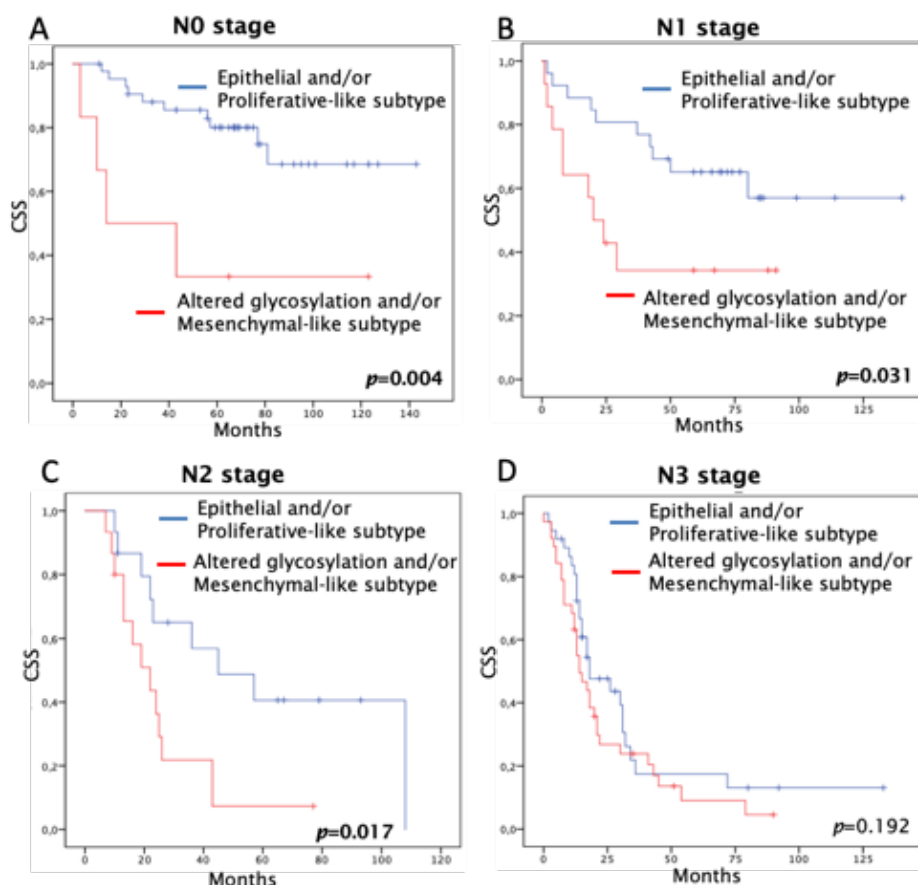


Figure 7. Cancer-specific survival (CSS) of patients composing groups C and D according with the N stage of the TNM staging system. Discriminated CSS of groups C and D in (A) N0, (B) N1, (C) N2 and (D) N3 tumours. Group C patients only have significantly decreased CSS when diagnosed with N0 ($p=0.004$), N1 ($p=0.031$) and N2 ($p=0.017$) grades of lymph-node involvement.

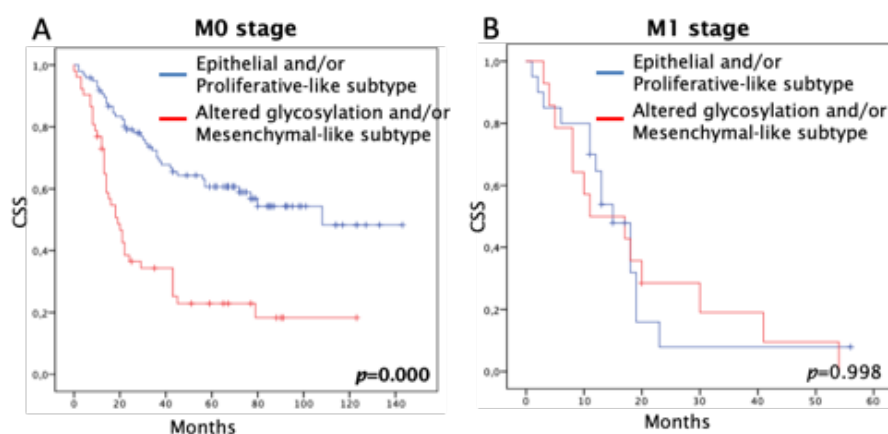


Figure 8. Cancer-specific survival (CSS) of patients composing groups C and D according with the M stage of the TNM staging system. Discriminated CSS of groups C and D in (A) M0 and (B) M1 tumours. Group C patients only have significantly decreased CSS when diagnosed with M0 ($p=0.000$) stage.

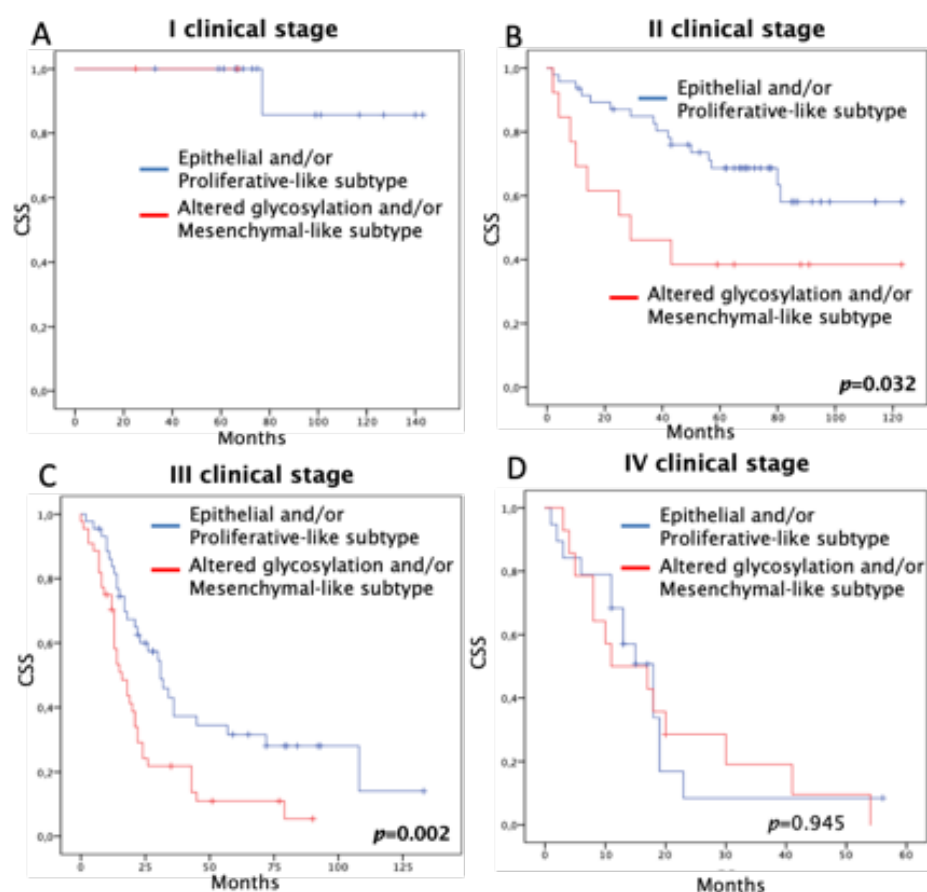


Figure 9. Cancer-specific survival (CSS) of patients composing groups C and D according with tumour clinical stage. Discriminated CSS of groups C and D in (A) I, (B) II, (C) III and (D) IV tumours. Group C patients only have significantly decreased CSS when diagnosed with stage II ($p=0.032$) or III ($p=0.002$) GC.

In summary, addressing molecular subtype of GC has significantly improved the stratification capability provided by classical clinicopathological variables (Lauren and Borrmann classifications, TMN). It was particularly useful for discriminating patient subgroups showing intermediate clinical histories. Moreover, it consistently demonstrated an association between epithelial-like phenotypes and better prognosis. In contrast, mesenchymal-like features, frequently associated with increased STn and SLe^A, was linked with worst survival. These observations set an important molecular rationale to improve patient stratification using molecular analysis. Moreover, it highlights the need to engage in a more comprehensive evaluation of the STn- and SLe^A-glycoproteomes associated with each molecular subtype towards precise GC targeting.

Concluding remarks and future perspectives

GC management remains challenging due to unprecise tumour classification based on classical clinicopathological features, which disregards the tremendous molecular heterogeneity presented by tumours of similar histological features. There is also an unmet need for efficient targeted and less-toxic therapeutics that may constitute valuable alternatives to chemotherapy, which currently constitutes the mainstay option. The introduction of molecular subtypes that classify tumours as Epstein-Barr virus, microsatellite unstable tumours, genomically stable tumours, chromosomal instability, has been introduced to overcome this limitation. Nevertheless, it has not yet provided targetable biomarkers for improving therapeutic outcomes.

Building on the substantial amount of data supporting a functional role for STn and SLe^A in GC we have included these glycans in comprehensive molecular models, exploiting well-known cancer biomarkers Ki-67 (for proliferation index) and E-cadherin (for evaluation of epithelial status).

We have concluded that STn and SLe^A antigens were widely expressed in GC, irrespectively of the type of disease. Moreover, they were present in the primary lesions as well as lymph node and distant metastasis and there are also suggestions that these antigens maybe present in circulating tumour cells in the blood. Moreover, STn overexpression was linked to lymph node involvement whereas the SLe^A antigen was associated with distant metastasis, in agreement with its known functional role in hematogenous dissemination [47, 51, 52]. Notably, the association of distinct glycoforms with different glycosylation routes may denote distinct glycan-mediated cancer dissemination pathways that warrant careful future evaluation. In addition, more studies are required to improve on the detection of circulating tumour cells carrying abnormal glycosylation and fully disclose the clinical implications of these findings for liquid biopsies. However, taken together with their restricted patterns in healthy tissues, these observations set critical foundations for targeted intervention. Moreover, the significant correlation between STn and SLe^A supports that

targeting both glycoforms could be an important milestone towards more effective glycan-based targeted therapeutics. Nevertheless, the precise mapping of the STn and SLe^A antigen is still challenged by potential off-target effects derived from the lack of total cancer-specificity, necessary to minimize potential off-target effects. In fact, our analysis of healthy tissues detected low amounts of both antigens in secreting cells of the gastrointestinal, colorectal and respiratory tracts, in accordance with previous reports [47, 53]. The observation of cancer and healthy tissues suggests that STn may be present in some minor subsets of B and T cells, as recently described [54].

To provide the necessary framework towards intervention we then evaluated these glycans in multi-biomarker settings, exploiting Ki-67 and E-cadherin, which are routine in most pathology laboratories. This led to a novel molecular panel composed of proliferation, epithelial differentiation and aberrant glycosylation markers capable of identifying worst prognosis patients. We observed that poor proliferative (low Ki-67 levels) and less cohesive (low E-cadherin expression) tumours displayed more aggressive traits and decreased CSS in comparison to patients showed more proliferative, epithelial-like lesions. Nevertheless, tumours presenting hybrid phenotypes in relation to proliferation and epithelial-character but presenting significant increase in altered glycosylation presented a survival close to the worst prognosis group, suggesting a critical role for glycosylation in GC aggressiveness, in agreement with the current state-of-the-art [55, 56]. The inclusion of these molecular phenotypes enabled the identification of worst prognosis patients across all current classical classification systems (Lauren, Borrmann, TMN system and clinical stage), highlighting the importance of tumour molecular characterization. Future studies should focus on increasing the discrimination capacity of our molecular marker by including information on micro-satellite instability, viral and p53 status. This will be crucial for engaging in more comprehensive targeted glycoproteomics studies that may lead to personalized targetable cancer glyconeantigens.

In summary, this work sets the foundations for including glycans in GC molecular subtyping, which warrants more comprehensive developments with the inclusion of other relevant biomarkers. It also demonstrated the importance of developing glycan-based targeted therapeutics, suggesting that targeting simultaneously STn and SLe^A may help increasing clinical response compared to monotherapies. Moreover, a combined strategy may allow abrogating the two main metastatic pathways involving regional lymph-nodes and hematogenous dissemination and, potentially, target circulating tumour cells. Future studies should focus improving the stratification power of the proposed molecular panel and disclosing the major carriers of these glycoepitopes envisaging bi-specific antibodies for targeted therapeutics and precision oncology.

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Table 2- Correlation between antigen expression and Borrmann classification

		Borrmann Classification				<i>p</i> value
		I n(%)	II n(%)	III n(%)	IV n(%)	
Ki-67	Low	4 (44.4)	34 (45.9)	22 (55.0)	32 (58.2)	0.518
	High	5 (55.6)	40 (54.1)	18 (45.0)	23 (41.8)	
E-Cadherin	Low	3 (33.3)	27(36.5)	21 (52.5)	30 (56.6)	0.095
	High	6 (66.7)	47 (63.5)	19 (47.5)	23 (43.4)	
STn	Low	8 (88.9)	57 (75.0)	34 (81.0)	39 (75.0)	0.710
	High	1 (11.1)	19 (25.0)	8 (19.0)	13 (25.0)	
SLe^A	Low	5 (55.6)	30 (39.5)	15 (35.7)	22 (38.6)	0.744
	High	4 (44.9)	46 (60.5)	27 (64.3)	35 (61.4)	

Table 3- Correlation between antigen expression and Lauren classification

		Lauren's Classification			<i>p</i> value
		Intestinal n (%)	Diffuse n (%)	Mixed n (%)	
Ki-67	Low	56 (46.7)	29 (63.0)	10 (47.6)	0.160
	High	64 (53.3)	17 (37.0)	11 (52.4)	
E-cadherin	Low	46 (37.7)	25 (55.6)	11 (52.4)	0.082
	High	76 (62.3)	20 (44.4)	10 (47.6)	
STn	Low	93 (75.0)	39 (84.8)	14 (66.7)	0.219
	High	31 (25.0)	7 (15.2)	7 (33.3)	
SLe^A	Low	47 (37.9)	16 (32.0)	12 (54.5)	0.191
	High	77 (62.1)	35 (68.0)	10 (45.5)	

Table 4. Correlation between antigen expression and T stage of the TNM staging system

		T stage				<i>p</i> value
		1 n(%)	2 n(%)	3 n(%)	4 n(%)	
Ki-67	Low	4 (33.3)	9 (60.0)	50 (49.0)	35 (57.4)	0.374
	High	8 (66.7)	6 (40.0)	52 (51.0)	26 (42.6)	
E-Cadherin	Low	3 (25.0)	5 (33.0)	42 (41.6)	35 (58.3)	0.057
	High	9 (75.0)	10 (66.7)	59 (58.4)	25 (41.7)	
STn	Low	11 (91.7)	12 (70.6)	80 (76.2)	45 (77.6)	0.592
	High	1 (8.3)	5 (29.4)	25 (23.8)	13 (22.4)	
SLe^A	Low	6 (50.0)	6 (35.3)	37 (35.2)	27 (42.9)	0.633
	High	6 (50.0)	11 (64.7)	68 (64.8)	36 (57.1)	

Table 5- Correlation between antigen expression and the N stage of the TNM staging system

		N				<i>p</i> value
		0 n(%)	1 n(%)	2 n(%)	3 n(%)	
Ki-67	Low	17 (34.7)	21 (55.3)	18 (60.0)	42 (58.3)	0.047
	High	32 (65.3)	17 (44.7)	12 (40.0)	30 (41.7)	
E-Cadherin	Low	15 (31.3)	13 (34.2)	19 (65.5)	38 (52.8)	0.007
	High	33 (68.8)	25 (65.8)	10 (34.5)	34 (47.2)	
STn	Low	45 (91.8)	33 (82.5)	20 (64.5)	49 (69.0)	0.007
	High	4 (8.2)	7 (17.5)	11 (35.5)	22 (31.0)	
SLe^A	Low	21 (42.9)	16 (40.0)	14 (43.8)	25 (33.3)	0.651
	High	28 (57.1)	24 (60.0)	18 (56.3)	50 (66.7)	

Table 6- Correlation between antigen expression and M stage of the TNM staging system

		M		<i>p</i> value
		M0 n (%)	M1 n (%)	
Ki-67	Low	73 (50.0)	18 (58.1)	0.269
	High	73 (50.0)	13 (41.9)	
E-cadherin	Low	67 (46.5)	13 (38.2)	0.248
	High	77 (53.5)	21 (61.8)	
STn	Low	117 (79.6)	26 (76.5)	0.422
	High	30 (20.4)	8 (23.5)	
SLe^A	Low	66 (44.0)	7 (19.4)	0.005
	High	84 (56.0)	29 (80.6)	

Table 7- Correlation between antigen expression and GC clinical stage

		Clinical Stage				<i>p</i> value
		I n(%)	II n(%)	III n(%)	IV n(%)	
Ki-67	Low	8 (44.4)	25 (43.1)	47 (55.3)	18 (60.0)	0.341
	High	10 (55.6)	33 (56.9)	38 (44.7)	12 (40.0)	
E-Cadherin	Low	2 (11.1)	20 (35.1)	51 (60.7)	12 (36.4)	0.000
	High	16 (88.9)	37 (64.9)	33 (39.3)	21 (63.6)	
STn	Low	16 (88.9)	51 (85.0)	59 (69.4)	25 (75.8)	0.093
	High	2 (11.1)	9 (15.0)	26 (30.6)	8 (24.2)	
SLe^A	Low	10 (55.6)	23 (38.3)	38 (43.2)	6 (17.1)	0.020
	High	8 (44.4)	37 (61.7)	50 (56.8)	29 (82.9)	

Table 8- Correlation between antigen expression in tumor and correspondent metastasis

			Tumour		<i>p</i> value
			Low n(%)	High n(%)	
Metastasis	STn	Low	30 (83.3)	3 (21.4)	0.000
		High	6 (16.7)	11(78.6)	
	SLe ^A	Low	14 (70.0)	11 (31.4)	0.006
		High	6 (30.0)	24(68.6)	
	Ki-67	Low	15 (62.5)	7 (26.9)	0.012
		High	9 (37.5)	19 (73.1)	
	E-Cadherin	Low	17 (60.7)	7 (30.4)	0.030
		High	11 (39.3)	16 (69.6)	

CHAPTER 2

**GLYCOENGINEERED NANOPARTICLES FOR SELECTIVE DRUG
DELIVERY IN GASTRIC CANCER**

The information provided in this chapter is based in the following publications:

1. **Fernandes E.***, Ferreira D. *, Peixoto A., Freitas R., Relvas-Santos M., Palmeira C., Martins G., Barros A., Santos LL., Sarmento B., Ferreira JA. Glycoengineered nanoparticles enhance the delivery of 5-fluorouracil and paclitaxel to gastric cancer cells of high metastatic potential. International Journal of Pharmaceutics. 2019. Impact Factor: 4.21, Q1. Accepted for publication. doi:10.1016/j.ijpharm.2019.118646.

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Glycoengineered nanoparticles enhance the delivery of 5-fluorouracil and paclitaxel to gastric cancer cells of high metastatic potential

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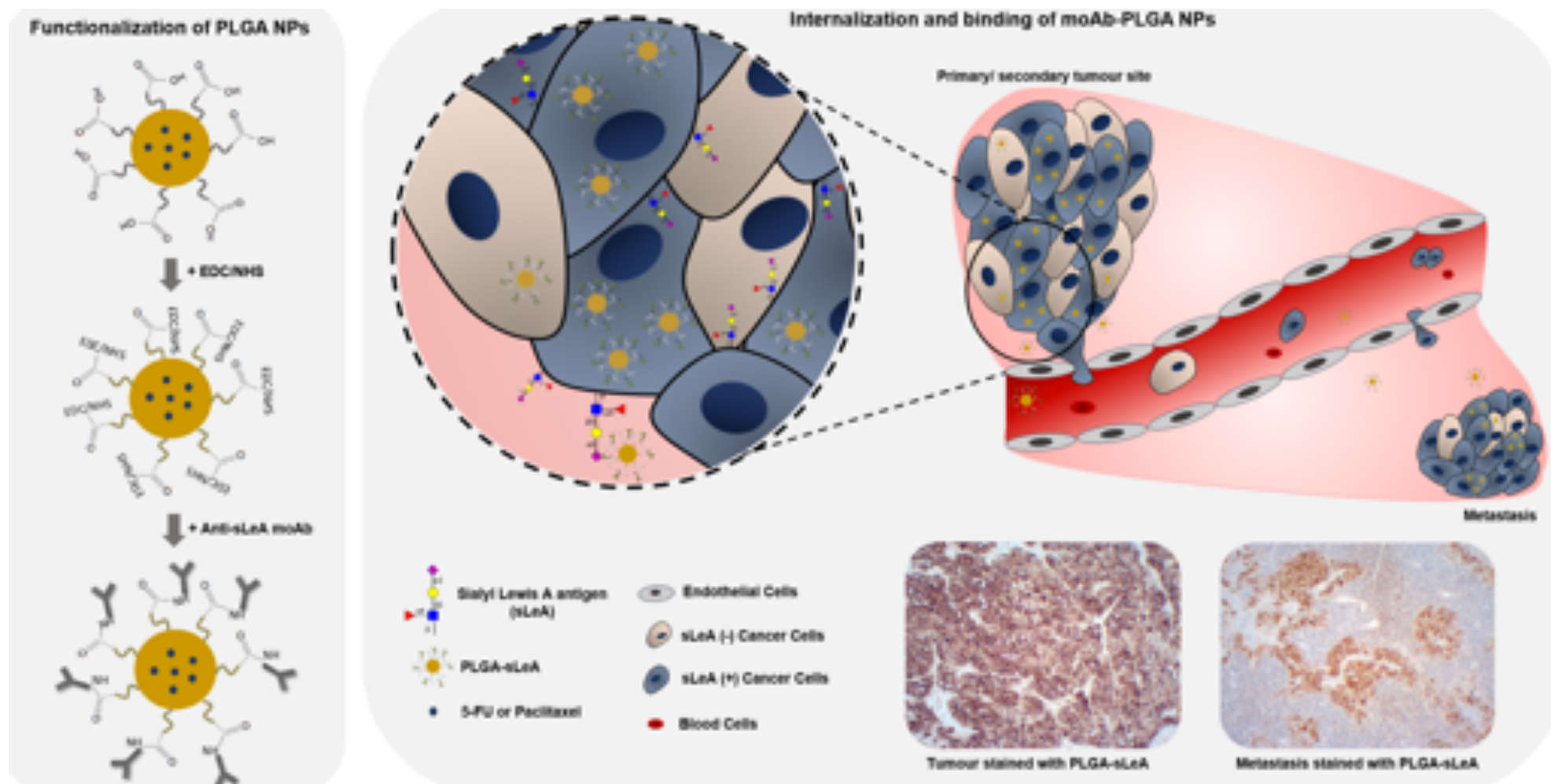
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Abstract

Gastric cancer is the third leading cause of cancer-related death worldwide, with half of patients developing metastasis within 5 years after curative treatment. Moreover, many patients cannot tolerate or complete systemic treatment due severe side-effects, reducing their effectiveness. Thus, targeted therapeutics are warranted to improve treatment outcomes and reduce toxicity. Herein, poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with 5-fluorouracil (5-FU) and paclitaxel were surface-functionalized with a monoclonal antibody targeting sialyl-Lewis A (SLe^A), a known glycan mediating hematogenous metastasis.

Nanoparticles, ranging from 137 to 330 nm, enabled the controlled release of cytotoxic drugs at neutral and acid pH, supporting potential for intravenous and oral administration. Nanoencapsulation also reduced the initial toxicity of the drugs against gastric cells, suggesting it may constitute a safer administration vehicle. Furthermore, nanoparticle functionalization significantly enhanced targeting to SLe^A cells *in vitro* and *ex vivo* (over 40% in comparison to non-targeted nanoparticles). In summary, a glycoengineered nano-vehicle was successfully developed to deliver 5-FU and paclitaxel therapeutic agents to metastatic gastric cancer cells. We anticipate that this may constitute an important milestone to establish improved targeted therapeutics against gastric cancer. Given the pancarcinomic nature of the SLe^A antigen, the translation of this solution to other models may be also envisaged.

Keywords: Gastric Cancer; Targeted Therapeutics; Drug Delivery; Poly(lactic-co-glycolic acid) Nanoparticles; Sialyl Lewis A.

Introduction

Gastric cancer is the third leading cause of cancer-related death worldwide, with an estimated 783,000 deaths in 2018 [382]. Locally advanced tumours are commonly subjected to repeated cycles of chemotherapy to reduce tumor size, risk of metastization and control disease dissemination [69]. However, half of patients develop metastasis within 5 years after curative treatment and many cannot tolerate or complete systemic treatment due to severe treatment side-effects, which may include neuropathy, cardiopathy, immune depression and many other severe outcomes [69]. Chemotherapy for gastric cancer frequently includes the intravenous administration of formulations containing 5-fluorouracil (5-FU), a fluorinated pyrimidine uracil analog whose mechanism of action is not yet fully understood [383]. Notwithstanding, it is known that 5-FU requires multiple conversions to form active metabolites, with the main cytotoxic activity being associated to thymidylate synthase binding and inhibition, ultimately interfering with nucleic acids synthesis [384]. Taxanes, particularly paclitaxel, have also emerged as important chemotherapeutic agents [67]. Paclitaxel targets the tubulin beta-subunit of microtubules, promoting defects in mitotic spindle assembly, chromosome segregation, and ultimately cell division [385]. Furthermore, it induces Bcl-2 phosphorylation, triggering apoptosis [386]. Although highly efficient against the tumour bulk it is also extremely hydrophobic, significantly limiting its solubilization and intravenous administration, urging the introduction of alternative and more effective delivery vehicles [387]. In summary, despite upfront improvements in patient survival, the efficacy of these chemotherapy agents for gastric cancer is constantly challenged by significant intra-tumoral clonal heterogeneity associated to multiple intrinsic and acquired resistance mechanisms and dose-limiting side-effects [388]. Thus, targeted therapeutics are warranted to improve treatment outcomes and reduce toxicity. Particular emphasis should be set on enhancing drug delivery to sub-populations of cancer cells showing higher metastatic potential, which are ultimately responsible for disease progression and

dissemination [374]. Nanoscale drug delivery vehicles have improved the efficacy of chemotherapeutic agents, with several formulations being available for different tumor types [389]. Namely, nanoparticles (NPs) for drug delivery increase the circulation time of the conjugated or entrapped therapeutic drugs by exploiting the tortuous and poorly differentiated vasculature of solid tumors that, in contrast to healthy tissues, allow the extravasation of drugs with sizes up to several hundred nanometers [390]. Solid tumors also lack functional lymphatic systems, making them unable to eliminate nanomaterials. Consequently, long-circulating nanomedicines tend to accumulate in tumors over time, a mechanism known as enhanced permeability and retention effect [391, 392]. Moreover, nanomedicines have improved the pharmacokinetics and efficacy of bioactive agents, while reducing systemic toxicity [393, 394]; nevertheless, few studies explored their potential to treat gastric cancer in comparison to other models [143]. In particular, poly(lactic-co-glycolic acid) (PLGA) is one of the most biocompatible polymers for NPs formulation envisaging controlled drug release. Its attractive properties include: (i) biodegradability and biocompatibility; (i) well established formulations and production methods adapted to various types of small or macromolecules of either hydrophilic or hydrophobic nature; (ii) approval by EMA and FDA, (iv) protection of drugs from degradation, (v) controlled release, (vi) possibility to graft the surface with different functional groups and/or biomolecules capable of providing stealthiness and/or improving interaction with biological materials/milieus and (vii) possibility to target NPs to specific cells and organs, significantly improving its efficiency [395]. Namely, PLGA NPs surface has been functionalized with antibodies and antibody fragments targeting relevant cancer-associated antigens such as CEA, HER2, and CD44 [396-398], ultimately demonstrating that antibody-functionalization potentiates the affinity of nanomedicines to cancer cells and significantly increases its retention in tumours. Therefore, given the careful choice of the target ligand, the development of more effective target-driven nanotherapeutics for gastric cancer may be feasible.

Malignant transformation is accompanied by significant alterations in cell surface glycosylation holding tremendous potential for cancer detection and targeted therapeutics [82, 83]. It has been long described that advanced stage gastric tumors and its metastasis overexpress the sialyl-Lewis A (SLe^A; CA19-9) antigen, a terminal epitope of glycan chains in proteins and lipids [207]. The SLe^A antigen mediates the adhesion of cancer cells to P- and E-selectin on endothelial cells, facilitating their intravasation into the bloodstream, dissemination and homing to distant locations [83, 207]. Furthermore, patients presenting strong SLe^A expression are at greater risk of developing distant metastasis and the presence of this antigen has been frequently observed in gastric metastasis [199, 399]. Moreover, since the SLe^A antigen is present at the cell-surface, thus easily accessible to antibodies, it constitutes an ideal target for drug delivery. As such, the present work devotes to the development of a novel biocompatible PLGA-based nanoparticle targeting aggressive SLe^A positive gastric cancer cells to deliver safely chemotherapy, according tumour staging and patient performance status.

Material and Methods

Materials

The human gastric cancer cell line NCI-N-87 isolated from a liver metastasis and the gastric adenocarcinoma cell line AGS were purchased from ATCC. Cell culture materials included RPMI 1640+GlutaMAX™-I and OptiMEM medium, heat-inactivated FBS, and penicillin-streptomycin solution from Gibco, Life Technologies. The fructosyltransferase inhibitor 2-fluoro-L-fucose (2FF) was purchased from Santa Cruz Biotechnology. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from ThermoFisher Scientific. The surfactant Triton X-100, the fixative paraformaldehyde (PFA) and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich. Cytotoxic drugs 5-FU and Paclitaxel were acquired from Sigma-Aldrich and Indena S.p.A, respectively. Reagents for nanoparticle preparation, such as ethyl acetate, pluronic F-127, acetone, 1-ethyl-3(3-

dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES), Coumarin-6 (C6) and sodium chloride were purchased to Sigma-Aldrich. Furthermore, 50:50 (Lactide:Glycolide) - Poly(Lactide-co-Glycolide) (PLGA) was provided by Corbion Biomaterials. Supernatants quantification was achieved using the Quick Start Bradford 1 x Dye Reagent from BioRad. Antibodies for nanoparticle functionalization and cellular staining, such as anti-SLe^A monoclonal antibody [CA19-9-203] and mouse IgG1 [MOPC-21] isotype control were provided by Abcam, while the polyclonal rabbit anti-mouse immunoglobulins/FITC secondary antibody was provided by DAKO. CellMask™ Orange 0.5x cell staining was acquired from Invitrogen. Enzymatic controls as Neuraminidase from *Clostridium perfringens* and PNGase F from *Elizabethkingia meningoseptica* were provided by Sigma-Aldrich. Tissue sections analysis was achieved using the Novolink Max Polymer DS Kit from Leica and citrate buffer from Vector.

Nanoparticle Production, Functionalization and Characterization

Nanoparticles Production

NPs containing 5-Fluoruracil (5-FU, Sigma-Aldrich) were generated by a modified water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation technique [400, 401]. Briefly, 30 mg of 50:50 (Lactide:Glycolide) - Poly(Lactide-co-Glycolide) (PLGA, Corbion-Purac Biomaterials) was dissolved in 4mL ethyl acetate (Sigma-Aldrich), following the addition of 10mg/mL 5-FU and emulsification at 70% amplitude for 30s using a Vibra-Cell™ ultrasonic processor in an ice bath. The second emulsion was achieved by adding 2% Pluronic F-127 (6mL) (Sigma-Aldrich) to the first emulsion, following Vibra-Cell™ ultrasonic homogenization for 60s and addition of the resulting mixture to 12mL of the same surfactant. Ethyl acetate evaporation from the final solution occurred for 5h under magnetic stirring at 250 rpm. Nanoparticles were isolated by centrifugation at 120 000 *g* for 40 min. Paclitaxel NPs were produced through a modified nanoprecipitation method [402]. Accordingly, 30 mg PLGA was dissolved in acetone (Sigma-Aldrich)

and added to 10 mg paclitaxel (Indena S.p.A). This organic solution was transferred through a 25 G needle to an aqueous solution of 1% Pluronic F-127 (15 mL) and solvent evaporations occurred under magnetic stirring at 250 rpm for 3h. A washing step with Milli-Q water was performed. Fluorescent PLGA NPs loaded with Coumarin-6 (C6) (Sigma-Aldrich) were obtained using the same methodology but dissolving 0.8 mg of C6 in acetone.

Nanoparticles functionalization with the anti-SLe^A antibody

PLGA NPs were covalently functionalized with an anti-SLe^A monoclonal antibody (CA19-9-203, ab222370, Abcam) following the carbodiimide chemistry for bioconjugation [397]. The coupling reaction was carried out in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich) allowing the carboxyl-terminated NPs to react with the primary amine of the antibody, yielding an amide bond. PLGA NPs were then centrifugated at 10,000*g* for 20 min and resuspended in 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich) pH 5.5. For NPs activation step, 1mL 0.1M EDC and 1mL 0.7M NHS were added dropwise to the previous NP suspension, which was kept 1h at room temperature under agitation in the dark. To remove excess EDC/NHS, PLGA NPs were centrifuged and resuspended in PBS pH 7.4 at a final concentration of 2 mg/mL. Activated NPs were then conjugated with 150μg anti-SLe^A moAb at 4°C overnight, centrifuged at 10,000*g* for 10min and re-dispersed in PBS at a final concentration of 4 mg/mL. The supernatants were stored for further antibody quantification by the Bradford method following the Quick Start Bradford 1 x Dye Reagent assay procedure (BioRad). The percentage of conjugation efficiency was determined according to the following equation:

$$\text{Conjugation efficiency (\%)} = \frac{\text{amount of inputted moAb} - \text{amount of antibody in supernatant}}{\text{amount of inputted moAb}} \times 100$$

In addition, the functionalization of PLGA NPs was evaluated by Fourier Transform Infrared Spectroscopy (FTIR) using an ABB MB3000 FTIR spectrometer (ABB) equipped with a MIRacle single reflection attenuated total reflectance (ATR) accessory (PIKE Technologies). For each NPs spectrum, a scan was collected in the mid- infrared region (3,600–600 cm⁻¹). Spectral analysis was executed using the Horizon MB™ FTIR software (ABB).

Nanoparticles physicochemical and morphological characterization

The size and polydispersity index (Pdl) of formulations were determined by Dynamic Light Scattering (DLS), while surface charge of NPs was determined by laser Doppler anemometry (LDA). After dispersion, NPs were diluted in 10 mM sodium chloride solution (pH 7.0) (Sigma-Aldrich) and DLS and LDA were performed with a Nano ZS Zetasizer (Malvern, Worcestershire, UK) at 25°C. Surface morphology and NPs size confirmation were performed by transmission electron microscopy (TEM) using a JEM-1400 microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Nanoparticles loading and controlled drug release

Association efficacy and drug loading

Supernatants of different formulations were analyzed by high-performance liquid chromatography (HPLC, Shimadzu UFLC Prominence System) to determine the association efficiency of 5-FU and Paclitaxel into PLGA NPs. The chromatographic conditions for 5-FU quantification included a Waters column with a guard column pumped at a flow rate of 0.5mL/min. The mobile phase was composed of acetonitrile and water (5:95, v/v) and the volume of sample injected was 10µl. The PDA detection wavelength was 265 nm with a run time of 7min at 20°C. The chromatographic conditions for Paclitaxel quantification were previously described by [397]. The Association Efficacy (AE) and Drug Loading (DL) were determined using the following equations:

$$AE (\%) = \frac{\text{Initial mass of drug} - \text{Mass of drug in supernatant}}{\text{Total mass of drug}} \times 100$$

$$DL (\%) = \frac{\text{Initial mass of drug} - \text{Mass of drug in supernatant}}{\text{Theoretical mass of nanoparticles}} \times 100$$

5-FU and paclitaxel controlled release

Envisaging both oral and intravenous administration of nanoformulations, the release profiles of 5-FU or Paclitaxel-loaded PLGA-NPs were determined in PBS and PBS 1% pluronic, respectively, at physiological pH 7.4 and gastric pH 3.5 under agitation at 37°C. Briefly, 500 µl of NPs suspensions supernatant were removed at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 32, 48, 56, and 72h and replaced with the same amount of respective buffer. The supernatants were centrifuged at 10,000g for 20min and quantified by HPLC as previously described.

Functionalized nanoparticles evaluation in gastric cancer cell models

Cell culture conditions

The human gastric cancer cell line NCI-N-87 isolated from a liver metastasis and the gastric adenocarcinoma cell line AGS were purchased from ATCC and cultured with RPMI 1640+GlutaMAX™-I medium (Gibco, Life Technologies), supplemented with 10% heat-inactivated FBS (Gibco, Life Technologies) and 1% penicillin-streptomycin (10,000 Units/mL penicillin; 10,000 mg/mL streptomycin; Gibco, Life Technologies). Cell lines were cultured as a monolayer at 37°C in a 5% CO₂ humidified atmosphere and were routinely subcultured after trypsinization. For internalization assays, 500 µM 2-fluoro-L-fucose (2FF, Santa Cruz Biotechnology) were added to NCI-N-87 cell culture medium for 24h to selectively inhibit fucosylation and consequently SLe^A levels at the cell-surface. This inhibition provided a negative control for *in vitro* PLGA-SLe^A NPs internalization assays.

Evaluation of SLe^A expression in gastric cancer cell models

PFA-fixed NCI-N-87 and AGS cells were screened for SLe^A using a 1:100 dilution of the anti-SLe^A [CA19.9-9-203] monoclonal antibody (ab116024, Abcam) in PBS 2% FBS for 1h at room temperature. Experiments using a mouse IgG1 [MOPC-21] Isotype Control (ab18443, Abcam) were included as negative controls. Polyclonal rabbit anti-mouse immunoglobulins/FITC (DAKO; F0313) was used as secondary antibody. Other experimental controls included the overnight digestion of whole cells with 10 mU/mL Neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) or 25mU/mL PNGase F from *Elizabethkingia meningoseptica* (Sigma-Aldrich) prior to flow cytometry analysis. PNGase F digestion contributed to disclosing the contribution of *N*-glycans for SLe^A expression on glycoproteins. Both controls consisted in overnight enzymatic digestion of 1×10^6 cells at 37°C under mild agitation. Two independent experiments were performed in triplicate for each cell line and condition. At least 20 000 events were acquired for each condition using a FC500 (Beckman Coulter) cytometer and data analysis was performed through InfinicytTM (Cytognos) Software.

Nanoparticles cytotoxicity profiles

The cytotoxicity of free drugs (5-FU and paclitaxel) and functionalized NPs loaded with chemotherapy were determined using MTT assay. Accordingly, gastric cancer cell lines NCI-N-87 and AGS were seeded into 96 well plates at a cell density of 40 000 and 10 000 cells/well, respectively, and exposed to increasing doses of free drugs and NPs formulations. After 24h exposure, conditioned media was replaced by 100µl fresh culture medium and 10 µL of a 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, ThermoFisher Scientific) stock solution was added to each well, following a 4h incubation at 37°C in a humidified chamber. 75 µl of conditioned medium was removed from the wells and 50 µL of DMSO (Sigma-Aldrich) was added to solubilize formazan crystals. Plates were incubated at 37°C for 10 minutes and absorbances were read at 540 nm in a microplate spectrophotometer. Positive controls (untreated

cells; empty functionalized NPs exposed cells) and a negative control (cells treated with 1% triton-X 100) were included. Thus, the metabolic activity (%) of living cells was calculated through the following the equation:

$$\text{Metabolic activity (\%)} = \frac{\text{absorbance of treated cells}}{\text{absorbance of positive control}} \times 100$$

Nanoparticles internalization by SLeA positive gastric cancer cells

The interactions between NPs and gastric cancer cell lines (NCI-N-87 and AGS) was evaluated by fluorescence microscopy and flow cytometry, using coumarin 6 (C6)-loaded NPs. NCI-N-87 cells exposed to 2FF (as described in section 2.6) and showing reduced SLe^A expression were used as negative controls. For fluorescent microscopy assays, cells were seeded in 12-well plates and incubated for 4h at 37°C with fresh medium or 1.5mg/mL PLGA NPs, PLGA NPs loaded with C6 (PLGAc), PLGAc-EDC/NHS, and PLGAc-SLe^A in OptiMEM. medium. Cells were fixed with 4% PFA and stained with CellMask™ Orange 0.5x (Invitrogen) for 6min at room temperature. The uptake of fluorescent NPs was evaluated under inverted fluorescence microscopy (Leica DMI 6000 from Leica), detecting PLGAc NPs through the GFP channel and CellMask™ stained cells through TXR the channel. Tlescan analysis was performed using the LAS X software. For flow cytometry analysis, cells were incubated according to the same protocol, detached through a non-enzymatic method and fixed with 4% PFA. PLGAc-NPs functionalized with Isotype IgG1 were added as negative control and cells were acquired in a NAVIOS cytometer (Beckman Coulter) using the Infinicyt™ (Cytognos) software. At least 20 000 events were acquired in each condition.

Functionalized nanoparticles binding to gastric tumours and healthy histological sections

Formalin-fixed paraffin embedded (FFPE) tissue sections from primary gastric lesions (3 advanced stage tumours; 3 superficial tumours) and corresponding metastasis, whenever available, from patients subjected to treatment at the Portuguese Institute of Oncology of Porto (IPO-Porto) in Portugal were elected for this study. All procedures were approved by the local ethics committee under patient's informed consent. Tumour sections were screened for SLe^A using the anti-SLe^A [CA19.9-9-203] mouse monoclonal antibody (ab116024, Abcam) and SLe^A functionalized PLGAc-NPs (PLGAc-SLe^A NPs) using the polymer method. Briefly, 3µm gastric cancer and healthy tissue (skin, lung, pancreas, colon, small intestine, liver, kidney, stomach) sections were deparaffinized, rehydrated and incubated for 15min with boiling citrate buffer (Vector). Tissue sections were then exposed to 3-4% hydrogen peroxide for 5min (Leica) and SLe^A was detected using the Novolink Max Polymer DS Kit (Leica) according to the manufacturer instructions. Positive and negative controls were tested in parallel. Negative controls consisted in 5% BSA devoid of primary antibody, PLGAc, PLGAc-EDC/NHS and PLGAc-Isotype NPs, while the positive control corresponded to a subsequent tissue section stained using the anti-SLe^A monoclonal antibody. An additional negative control was performed with recourse to 0.2U/mL Neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) overnight at 37°C prior incubation with anti-SLe^A moAb and PLGAc-SLe^A.

Statistical analysis

GraphPad Prism 7 analysis was used to analyse the significance level (ANOVA with Dunnet's test) and determine the EC50 value. All experiments were performed in triplicate and represented as mean ± standard deviations (SD). A Kruskal-Wallis one-way analysis of variance (one-way non-parametric ANOVA) was used to evaluate the effect of pH on drugs-controlled release. Two-way ANOVA with Bonferroni's post hoc test (GraphPad Prism software Inc., USA) was used to analyse cytotoxicity profiles. The level of significance

was set at probabilities of “*” $p < 0.05$; “**” $p < 0.01$; “***” $p < 0.001$; and “****” $p < 0.0001$.

Results and Discussion

The present work focusses on the development of novel biocompatible PLGA-based NPs targeting gastric cancer cells showing higher metastatic potential, translated by the expression of cell surface SLe^A antigen. The overall objective is to improve the delivery of 5-FU and paclitaxel to these cells while providing means to surpass systemic administration issues, such as indiscriminate cytotoxicity and potential off-target effects.

Physicochemical and morphological characterization of nanoparticles

PLGA NPs were produced through the double emulsion-solvent evaporation technique and modified nanoprecipitation method. Empty (controls) as well as 5-FU- and paclitaxel-loaded NPs with or without SLe^A moAb functionalization using an EDC/NHS linker were constructed and analyzed according to critical physicochemical and morphological properties, such as mean size, size distribution, surface charge, association efficiency (AE) and drug loading (DL) (**Table 1**).

Nanoparticle size, as determined by dynamic light scattering (DLS), ranged between 137 and 330 nm. Furthermore, transmission electron microscopy (TEM) confirmed the aspheric shape of NPs, as well as the size range (**Figure 1**). The size of the NPs determines its vascular and lymph systems clearance, as such, the optimal NP size to escape accelerated elimination was approximately 100nm [403].

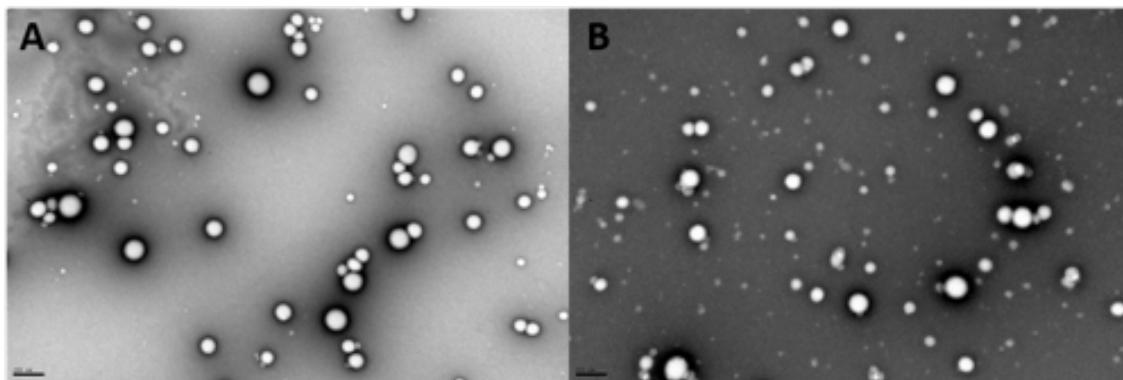


Figure 1. Transmission Electron Microscopy (TEM) analysis of 5-FU moAb functionalized PLGA-NPs (A) and Paclitaxel functionalized P-LGA NPs (B). Scale bar represents 200nm.

However, manipulation of surface characteristics provided an opportunity to generate ideal systems despite the increase in size resulting from functionalization. The increased size of functionalized NPs probably resulted from the addition of EDC/NHS linkers to its surface and/or NPs aggregation, which was described as a common event derived from carbodiimide chemistry [404]. In addition, FTIR analysis of functionalized NPs was performed to confirm the covalent binding of anti-SLe^A moAb to PLGA-NPs (**Figure 2**). Accordingly, at 1750 cm⁻¹, a marked peak suggested the presence of a carbonyl bond (C=O stretching vibration) characteristic of PLGA (**Figure 2A**). Antibody covalent conjugation to the NPs *via* an EDC/NHS linker led to the appearance of an amide bond (C=O stretching vibration) at 1640 cm⁻¹. Moreover, an additional peak at 1560 cm⁻¹ stood out in the PLGA-SLe^A NPs spectra, corresponding to the amine groups of the moAb (N-H bending vibrations) (**Figure 2C**). Together these findings confirm the covalent binding of the anti-SLe^A moAb to PLGA NPs. Furthermore, the polydispersity index (Pdl) of empty and non-functionalized NPs ensured a highly monodisperse population (<0.1), which was amplified by moAb functionalization [405]. These findings further indicate NPs aggregation as a result of the functionalization protocol. All formulations were negatively charged and within a desirable zeta potential for nanosuspensions stability, since in the case of a combined electrostatic and steric stabilization, a minimum zeta potential of ± 20 mV is desirable [406].

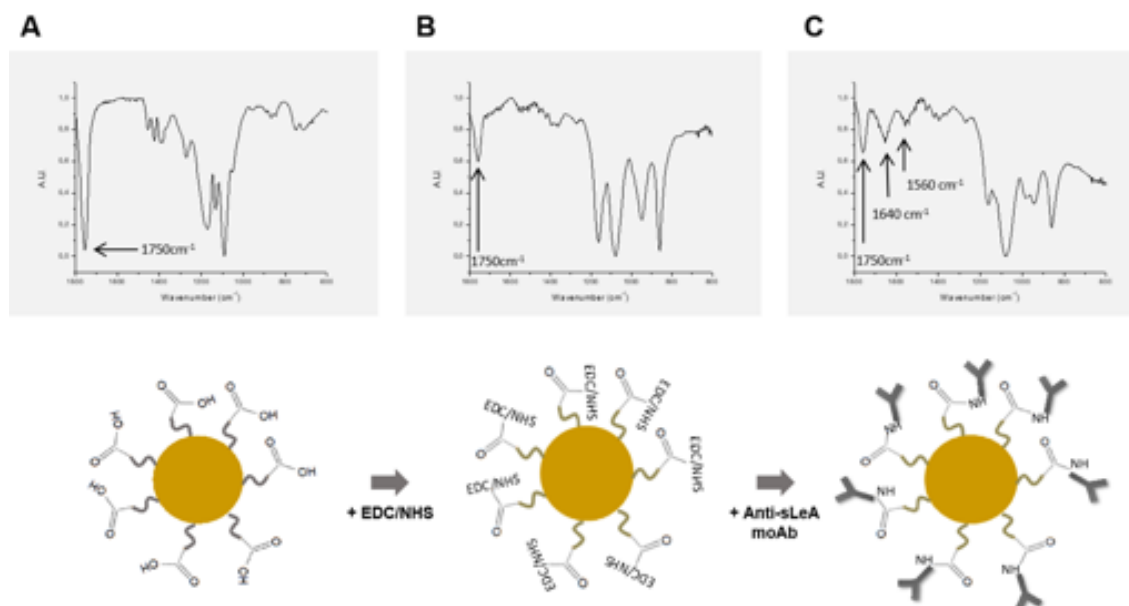


Figure 2. FTIR analysis of functionalized NPs. Spectra of PLGA NPs showing a marked peak at 1750 cm⁻¹ characteristic of PLGA (A); Spectra of PLGA-EDC/NHS NPs showing the no spectral alterations compared to PLGA NPs (B); Spectra of PLGA-SLe^a-NPs showing marked peaks at 1750 cm⁻¹, 1640 cm⁻¹ and 1560 cm⁻¹ vowing for the presence of carbonyl, amide and amine bonds corresponding to the covalent binding of anti-SLe^a moAb to PLGA-NPs (C).

Furthermore, mild negative NPs surface charges could prevent strong repulsion from the negatively charged cell membrane, while decreasing non-specific interactions and NPs spontaneous aggregation.

The association efficacy (EA) and drug loading (DL) of 5-FU and paclitaxel into PLGA-NPs was indirectly determined by HPLC. Accordingly, 5-FU EA ranged 40% while DL was around 14%. In turn, paclitaxel-loaded NPs exhibited AE values over 90% and DL around 30%. These findings are in accordance with the solubility profile of both drugs. Notably, the hydrophilicity of 5-FU increases the diffusion to the aqueous external phase during the encapsulation process, lowering its association efficiency. Contrastingly, the high hydrophobicity of paclitaxel favours encapsulation through the nanoprecipitation method, thereby enhancing AE and DL values [407].

To assess NPs functionalization, NPs formulation supernatants were analyzed by the Bradford method after anti-SLe^a moAb conjugation and the percentage of conjugation efficiency (CE) was determined (**Table 1**). Accordingly, the CE of the moAb to PLGA-NPs was 67%, exceeding the expected values for the carbodiimide chemistry of immunoconjugation [408].

Table 1. Properties of empty, non-functionalized and functionalized NPs, including the mean size, polydispersity index (Pdl), surface charge, association efficacy (EA) and drug loading (DL). The values are represented as mean values $\pm$ SD (n=3).

Encapsulated Agent	Nano-construct	Size (nm)	Pdl	Surface Charge (mV)	EA (%)	DL (%)	moAb CE (%)
5-FU	Empty	137 $\pm$ 0.2	0.058 $\pm$ 0.001	-7.1 $\pm$ 0.5	-	-	-
	Non-functionalized	216 $\pm$ 2.4	0.175 $\pm$ 0.007	-1.8 $\pm$ 0.4	40.5 $\pm$ 2.4	14.1 $\pm$ 0.9	-
	Non-functionalized-EDC/NHS	224 $\pm$ 1.6	0.131 $\pm$ 0.004	-2.9 $\pm$ 0.5	-	-	-
	Functionalized	330 $\pm$ 5.9	0.403 $\pm$ 0.029	-0.9 $\pm$ 0.1	39.4 $\pm$ 1.5	13.9 $\pm$ 0.3	-
Paclitaxel	Empty	174 $\pm$ 1.1	0.099 $\pm$ 0.043	-6.3 $\pm$ 0.5	-	-	-
	Non-functionalized	202 $\pm$ 5.3	0.152 $\pm$ 0.004	-4.1 $\pm$ 0.9	96.6 $\pm$ 1.2	32.0 $\pm$ 0.1	-
	Non-functionalized-EDC/NHS	224 $\pm$ 1.	0.131 $\pm$ 0.004	-2.9 $\pm$ 0.5	-	-	-
	Functionalized	301 $\pm$ 7.6	0.390 $\pm$ 0.013	-0.9 $\pm$ 0.1	90.5 $\pm$ 1.1	29.8 $\pm$ 0.4	-
Coumarin-6	Functionalized	328 $\pm$ 1.0	0.491 $\pm$ 0.061	-14.1 $\pm$ 0.3	-	-	67 $\pm$ 3.0

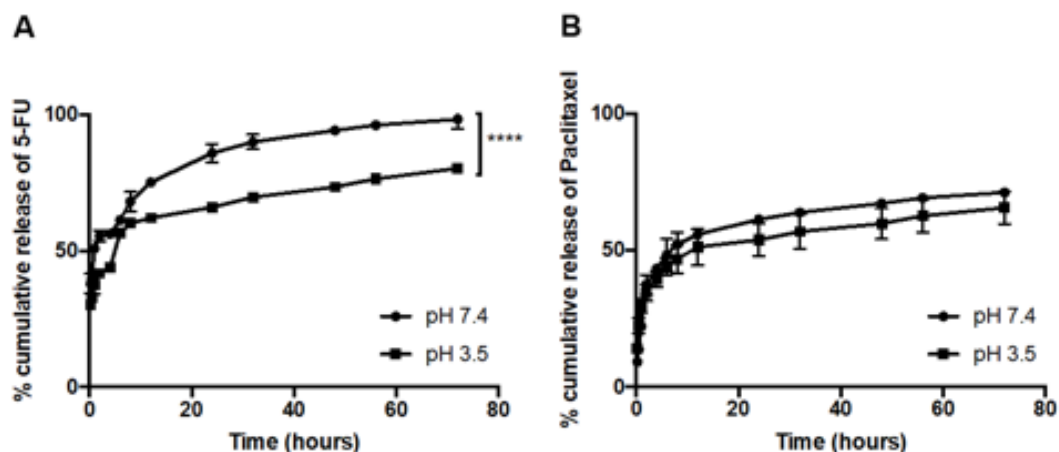


Figure 3. Cumulative percentage release 5-FU (A) and Paclitaxel (B) from loaded PLGA-NPs throughout 72h at pH 7.4 (circle) and pH 3.5 (square). At any given timepoint, 5-FU-NPs released significantly higher amounts of cytotoxic drug at pH 7.4 than at 3.5, vowing for a pH-dependent release. Contrastingly, Paclitaxel release from PLGA-NPs was not pH-dependent in this experimental setup. Values are represented as a mean $\pm$ SD, $n = 3$. “****” $p < 0.0001$ (Kruskal-Wallis test)

5-FU and paclitaxel release study in aqueous solution

Envisaging the intravenous but also the oral administration of nanoformulations, the release profiles of 5-FU or paclitaxel-loaded PLGA-NPs were determined by HPLC at the physiological pH 7.4 and at the gastric pH 3.5 (**Figure 3**).

Both cytotoxic drugs experience a burst release in the first few hours, followed by slower and more controlled release. Moreover, at any given timepoint, 5-FU-NPs released significantly higher amounts of cytotoxic drug at pH 7.4 than at 3.5 ($p < 0.001$), vowing for a pH-dependent release. Contrastingly, paclitaxel release from PLGA-NPs was not pH-dependent in this experimental setup. Furthermore, 5-FU release occurred at an accelerated pace compared to paclitaxel, which could be once again due to its hydrophobicity and resistance to diffusion to the aqueous environment. As such, PLGA-NPs may offer an opportunity for the controlled release of paclitaxel *in vivo* and support the possibility of intravenous as well as oral administration.

SLe^A expression in Gastric cell lines for *in vitro* studies

Two gastric cancer cell lines of different origins, namely the human gastric adenocarcinoma cell line AGS and human gastric metastatic cell line NCI-N87 were screened for SLe^A expression by flow cytometry, envisaging models for screening NPs toxicity profile and affinity. Autofluorescence, secondary antibody and IgG1 isotype controls were used to define positivity thresholds. Enzymatic negative controls using α -Neuraminidase and PNGase F, removing sialic acids from SLe^A impairing antibody recognition and removing *N*-glycan chains potentially carrying SLe^A, respectively, were also included. According to this analysis, the epithelial cell line AGS does not show significant SLe^A expression, while metastatic NCI-N-87 cells overexpress this antigen. Furthermore, in NCI-N-87 cells, SLe^A is mostly represented in *N*-glycans as demonstrated by the dramatic signal decrease upon PNGase F treatment (**Supplementary Figure 1**). These results are consistent with other recently published studies demonstrating the increased expression of *FUT3* and *ST3GAL4* genes in NCI-N-87 cells compared to AGS cells, which translates in increased terminal α 1,4-linked fucosylation and α 2,3-linked sialylation in terminal glycan chains, ultimately leading to SLe^A overexpression [121]. Furthermore, the presence of high-levels of SLe^A in NCI-N-87 cells, originally isolated from a liver metastasis, reinforces this glycan's role in P- and E-selectin mediated hematogenous dissemination [409].

Nanoparticle cytotoxicity profiles *in vitro*

The cytotoxicity of free and nanoencapsulated chemotherapy agents were determined using the metabolic MTT assay, a colorimetric assay assessing cell metabolic activity and reflecting the number of viable cells. Untreated cells and cells exposed to empty functionalized NPs (Empty NP moAb) were included as positive controls, while cells treated with 1% triton-X 100 that promotes massive cell death were included as negative control. Gastric cancer cell lines NCI-N-87 (SLe^{A+}) and AGS (SLe^{A-}) were exposed to increasing concentrations of free and targeted nanoencapsulated drugs (10

- 100 nM), including the half maximal inhibitory concentration (IC₅₀) of both drugs (1.6 and 60 nM for 5-FU in NCI-N-87 and AGS, respectively; 7nM and 23 nM for paclitaxel in NCI-N.87 and AGS, respectively) [410-412] (**Figure 4**). Two-way ANOVA showed that drug concentration, the type of formulation (free drug, empty functionalized NPs, antibody-NP conjugates) and the synergism of both factors contributed to influence cell viability ($p < 0.0001$), irrespectively of the cell line. Moreover, the increase in concentration of free drugs was inversely correlated with cellular viability in both cell lines. Nevertheless, AGS were more sensitive to 5-FU in comparison to paclitaxel whereas NCI-N-87 presented the opposite behaviour, denoting a cell-dependent drug response profile. However, in general, the AGS cell model was more sensitive to the tested chemotherapy agents in comparison to NCI-N-87, irrespectively of the drug. Contrastingly, nanoencapsulation significantly reduced the toxicity profile of both drugs for the two cell lines. Moreover, the fact that the NPs content had not been entirely released after 24h further reinforces the notion of controlled release provided by nanoencapsulation and may account for the lower toxicity presented by this delivery strategy. Notably, the targeted nature of nanoencapsulated drugs did not translate into noticeable differences in cytotoxicity between cell lines at 24h, irrespectively of marked differences in SLe^a expression (Figure 4). Such observations may be directly linked to the slower drug release kinetics promoted by nanoencapsulation and supported by controlled release studies. Moreover, it demonstrates that the presence of the antibody did not promote toxicity. Overall, cell viability for all nanoformulations was significantly above 70%, which is regarded as toxicity threshold according to ISO 10993-5 [413], reinforcing the value of these NPs for drug delivery.

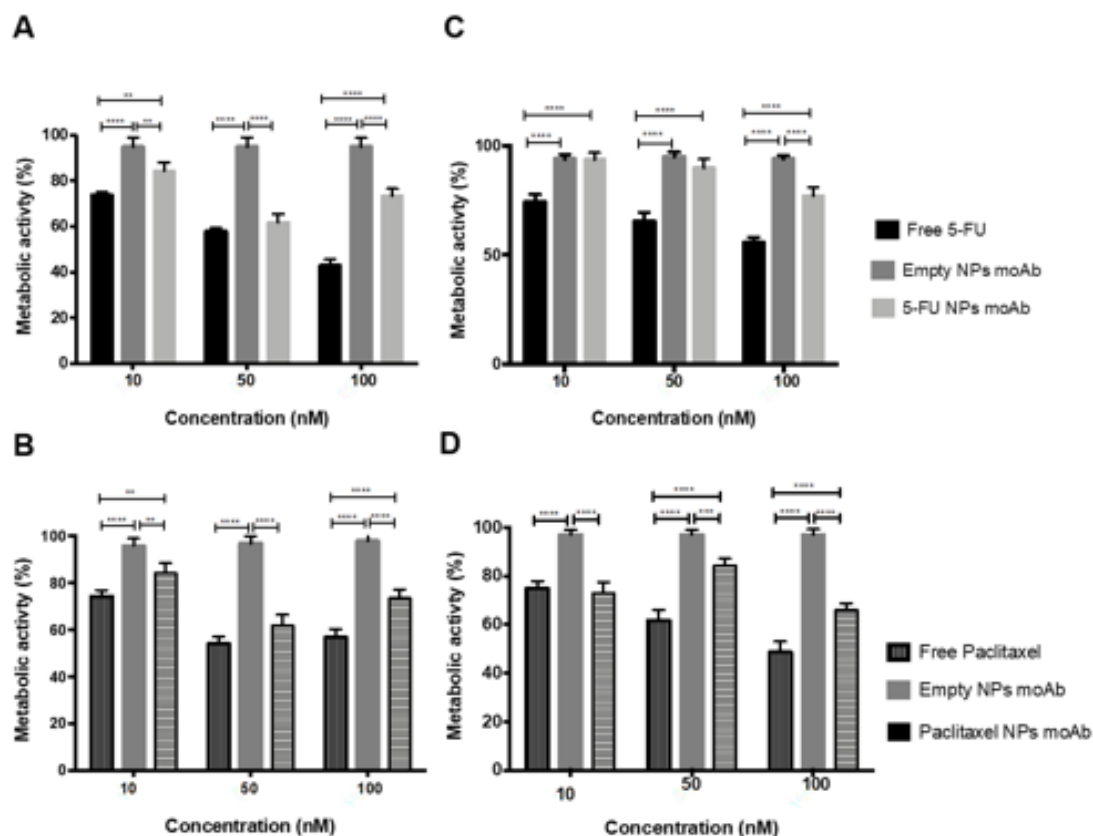


Figure 4. Metabolic activity of gastric cancer cell lines AGS (**A, B**) and NCI-N-87 (**C, D**) after 24h exposure to 10, 50 and 100 nM free 5-FU and Paclitaxel or SLe^a moAb functionalized loaded NPs. Empty functionalized NPs were added as negative control. The increase in concentration of free drugs was inversely correlated with cellular viability in both cell lines. AGS cells were more sensitive to 5-FU in comparison to paclitaxel whereas NCI-N-87 presented the opposite behaviour, denoting a cell-dependent drug response profile. Overall, AGS cells were more sensitive to the cytotoxic agents in comparison to NCI-N-87. Contrastingly, nanoencapsulation significantly reduced the toxicity profile of both drugs for the two cell lines. Values are represented as mean $\pm$ SD, $n = 3$. “**” $p < 0.01$; “***” $p < 0.001$; and “****” $p < 0.0001$ (Bonferroni’ test)

Internalization of nanoparticles by SLe^a positive cells

The interactions between NPs and gastric cancer cell lines (NCI-N-87 and AGS) was evaluated by fluorescence microscopy and flow cytometry, using Coumarin 6 (C6)-loaded NPs (**Figure 5**). According to FACS analysis, functionalized NPs showed almost a 2-fold increase in affinity for NCI-N-87 cells in comparison to void NPs, NPs conjugated with the ECD/NHS linker or the IgG1 isotype control, as translated by the increased mean fluorescence

intensity of coumarin-6 (**Figure 5A and B**). Contrastingly, this was not observed for AGS cells, reinforcing that the presence of the antibody significantly enhanced NPs uptake by cancer cells expressing SLe^A (**Figure 5A and C**). However, studies involving non-functionalized and IgG1 isotype-functionalized particles showed significantly higher NPs concentrations in AGS in comparison to NCI-N-87. Such observations suggest that the magnitude of the targeting effect may be underestimated by the initial unselective uptake behaviour demonstrated by AGS in comparison to NCI-N-87. As such, to elucidate further on the targeted effect of NPs, NCI-N-87 cells were incubated with 2FF, a known inhibitor of fucosyltransferases responsible by SLe^A biosynthesis [414], which resulted in a 20% decrease in the total amount of cell surface antigen and a 25% decrease in NPs internalization (**Supplementary Figure 2**).

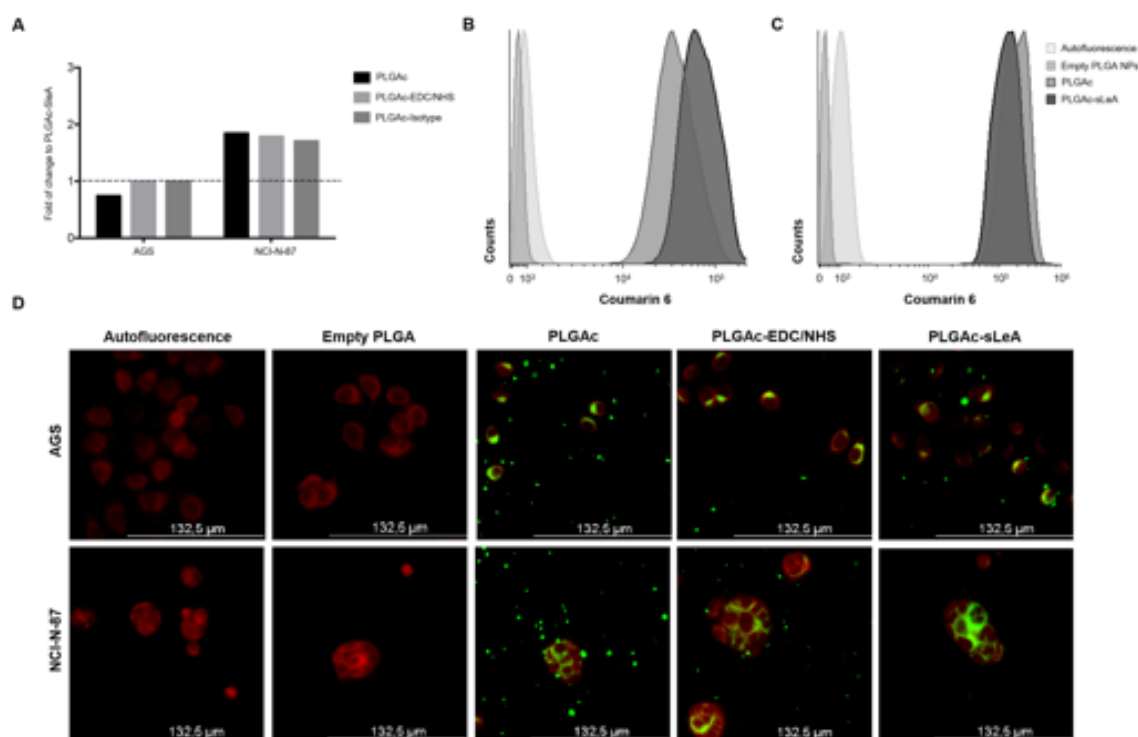


Figure 5. Interactions of functionalized PLGA coumarin 6 (C6)- loaded NPs with gastric cancer cell lines (NCI-N-87 and AGS). Functionalized NPs (PLGA-sLe^A) showed a 2-fold increase in affinity for NCI-N-87 cells compared to void NPs (PLGA), NPs with ECD/NHS linker (PLGA- ECD/NHS) or functionalized with IgG1 isotype control (PLGA-Isotype), as translated by the increased mean fluorescence intensity (**A, B**), which was not observed in AGS cells (**A, C**). Moreover, NCI-N-87 cells presented a higher uptake of functionalized NPs

per cell in comparison to non-functionalized controls, while AGS cells showed no difference in internalization rates (**D**).

Fluorescent immunocytochemistry analysis using CellMask™ staining and Coumarin 6-labelled NPs was performed to further assess NPs internalization by gastric cancer cells. NCI-N-87 cells presented a higher uptake of functionalized NPs per cell in comparison to non-functionalized controls (**Figure 5D**). The higher fluorescence intensity observed in the cytoplasm of NCI-N-87 cells exposed to functionalized NPs also suggested an increase in NPs uptake, thus in agreement with flow cytometry analysis. Again, AGS cells displayed no differences in internalization rates. These observations reinforce that antibody functionalization improved NPs targeting to SLe^A expressing cells, suggesting potential as a drug delivery vehicle for metastatic gastric cancer.

Binding of functionalized nanoparticles to gastric tumour and healthy tissues sections *ex vivo*

To disclose the *ex vivo* targetability of functionalized NPs, formalin-fixed paraffin embedded (FFPE) gastric tissue sections were first screened for SLe^A by immunohistochemistry using the free antibody and functionalized NPs. Three advanced stage primary lesions and corresponding distant metastasis as well as three superficial gastric tumours were elected for this study. High levels of SLe^A were observed in the tumour area of advanced cases and corresponding metastasis but not in the apparently normal adjacent mucosa (**Figure 6**). SLe^A was mostly detected at the cell surface, in accordance with its typical expression pattern in human tissues [186]. In addition, low SLe^A expression was observed in early stage lesions, reinforcing the aggressive nature of this molecular feature [415]. Notably, SLe^A was detected in all the metastasis, reinforcing its role in this process and suggesting potential for targeting disseminated disease [116, 186, 416]. Parallel analysis of the same sections with NPs functionalized with IgG1 isotype antibody, empty PLGA NPs and PLGA-EDC/NHS NPs showed no unspecific staining. Moreover, anti-SLe^A functionalized NPs

displayed a staining pattern similar to the free antibody. The specificity of NPs for cancer cells was reinforced by the existence of reactivity solely in tumour areas but not in non-neoplastic areas, including lymphoid and muscle tissues. This was further confirmed by the large reduction in NPs-mediated staining in neuraminidase treated tumour sections (data not shown). Interestingly, antibody functionalized NPs provided a more intense staining pattern compared to the free antibody, suggesting that NPs may have provided signal amplification as a result of multiple antibody conjugates per nanoparticle. Future studies should devote to disclose the potential of nanoplatforms in deciphering trace amounts of clinically relevant cancer-antigens in tumour sections.

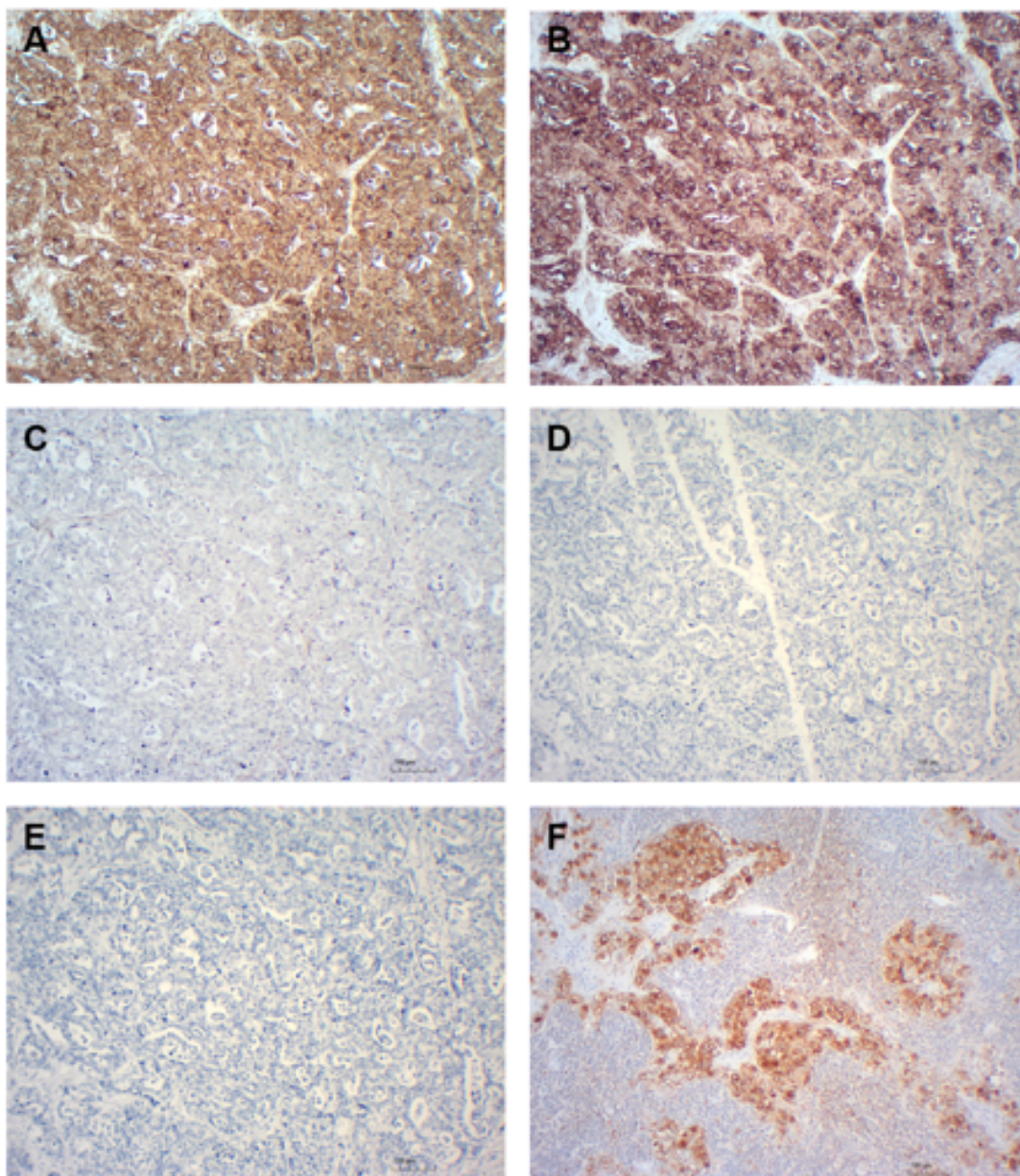


Figure 6. Immunohistochemistry analysis of gastric cancer tissue sections using an anti-SLe^a moAb (A) and SLe^a functionalized PLGA-NPs (PLGA-SLe^a NPs) (B). Negative controls using empty PLGA NPs (C), PLGA-EDC/NHS NPs (D) and PLGA-Isotype NPs (E) were run in parallel and showed no unspecific staining. High levels of SLe^a were observed at the cell surface of tumour cells in advanced cases and corresponding metastasis (F) but not in the apparently normal adjacent mucosa (data not shown).

Finally, we have assessed the affinity of functionalized NPs for a panel of healthy tissue sections from 9 relevant organs (skin, lung, stomach, small intestine, colon, liver, pancreas, kidney and lung) (**Figure 7**). Functionalized NPs did not bind to lymphoid and muscle tissues, as previously observed for gastric tumour adjacent mucosa. It also did not bind to vasculature or normal stomach epithelium, with the exception of a faint affinity for mucinous secretions known to yield high glycan density [186, 417].

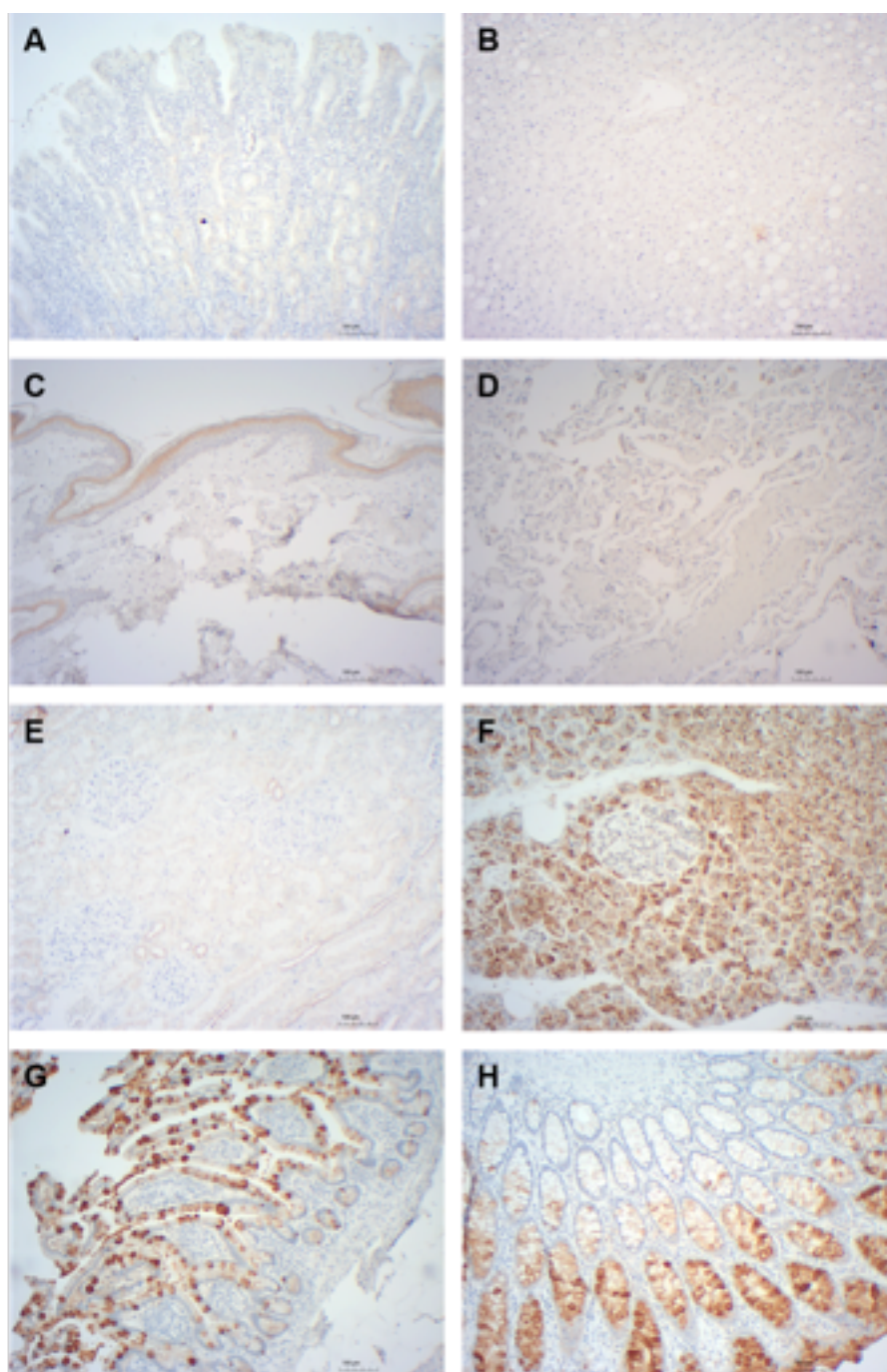


Figure 7. Immunohistochemistry analysis of healthy tissue sections from 9 relevant organs (stomach **(A)**, liver **(B)**, skin **(C)**, lung **(D)**, kidney **(E)**, pancreas **(F)**, small intestine **(G)** and colon **(H)**) using PLGA-SLe^A NPs. Functionalized NPs did not bind to leucocytes, muscle, stomach epithelium, liver or kidney sections. Low levels of staining were observed in pancreatic acini, superficial skin layers and in goblet cells.

Moreover, NPs did not show any affinity for liver and kidney sections; nevertheless, low levels of staining could be observed in the exocrine pancreas (pancreatic acini) and in mucus-producing cells, particularly in goblet cells of the intestinal and respiratory epithelia. A faint cytoplasmatic staining could also be observed in the skin upper layers (granular and lucidium layers) but no expression was found in the other four epidermal layers. Taken together, these observations demonstrate that functionalized NPs present a restricted affinity for healthy tissues and high affinity for SLe^A expressing cancer cells in *ex vivo* settings. This emphasises the potential of this nanomedicine for drug delivery in complex tissue contexts and sets the necessary molecular rational for studies *in vivo*.

Conclusions

Gastric cancer remains the third leading cause of cancer-related death worldwide due to difficulties controlling disease dissemination associated with the severe toxicity of chemotherapy agents, urging novel, less toxic and more effective target therapeutics. As such, the present work devoted to the development of a novel biocompatible PLGA nanoconstruct glycoengineered to target SLe^A expressing gastric cancer cells. This is expected to improve the safety related with the delivery of chemotherapy to cancer cells, with emphasis on those with increased metastatic potential. Moreover, targeting the SLe^A antigen, which plays a functional role in hematogenous dissemination of disease while presenting a very restricted expression pattern in healthy tissues, holds potential to reduce the off-target effects of current systemic chemotherapy. This aspect is particularly critical for improving the management of fragile gastric cancer patients unable to tolerate current chemotherapy formulations due to toxicity issues.

Envisaging this goal, we have synthesized nanoparticles with a size ranging between 137 and 330 nm upon functionalization. All formulations displayed acceptable polydispersity index and were within a desirable zeta potential for nanosuspensions stability for future clinical applications. The association efficacy of 5-FU and Paclitaxel were 40% and 90%, respectively, reflecting their dissimilar affinity for the external phase during the encapsulation processes [407]. Furthermore, the conjugation efficiency of the anti-SLe^A monoclonal antibody at the PLGA-NPs surface was 67%, exceeding previous reports exploiting carbodiimide chemistry for immunoconjugation [408].

We have then accessed the controlled release of the two nanoencapsulated drugs at physiological and acid pH, envisaging venous and oral administrations. Accordingly, 5-FU diffusion into solution was favoured by the physiological pH, while paclitaxel was found not to be pH-dependent. In addition, 5-FU release occurred at an accelerated pace compared to Paclitaxel but none of the cytotoxic agents was fully transferred to solution after 24h. As such, PLGA-NPs may offer an opportunity for the controlled release of both therapeutic agents *in vivo*.

For studies *in vitro* we elected the AGS and NCI-N-87 gastric cancer cell lines, presenting distinct molecular and pathological natures. Flow cytometry analysis revealed striking differences regarding SLe^A expression. Namely, epithelial AGS cells did not express SLe^A, while the hepatic type metastatic NCI-N-87 cells overexpressed the antigen. These findings were supported by previous reports describing that NCI-N-87 cells overexpress key glycosyltransferases responsible by SLe^A overexpression [121]. It is also consistent with the origin of NCI-N-87 cells (liver metastasis) as well as the SLe^A antigen functional role in metastasis development. These cell lines were first used to compare the cytotoxicity of encapsulated drugs with the free agents for a short initial exposure periods (24h). These assays demonstrated that nanoencapsulation significantly reduced the toxicity of both drugs due to the controlled release effect provided by the biocompatible polymer. Such observations are also in agreement with previous reports, supporting the excellent protective properties of nanoencapsulation and its potential for

reducing the toxicity of different types of drugs. This is particularly important to support the development of safer drug delivery systems. Toxicity assays *in vivo* are now required to set the rational for clinical translation. Moreover, in internalization assays *in vitro*, functionalized NPs demonstrated higher affinity for SLe^A expressing cells, supporting the relevance of antibody functionalization. This was later confirmed by *ex vivo* assays in gastric tumours and healthy tissues, emphasising the potential of this nanomedicine for drug delivery given its significant affinity to cancer cells compared to healthy tissues. Future studies should now focus on translating these findings into pre-clinical tests in relevant animal models, namely by exploiting patient derived xenografts. Emphasis should also be set on disclosing the nature of the glycoproteins carrying the SLe^A envisaging the design of even more specific ligands for cancer cells. Finally, given the pancarcinomic nature of the SLe^A antigen we anticipate that the success of this approach might be translatable to other tumour types, including pancreas [418], breast [419, 420], oesophageal [421], colorectal [422, 423], bladder [424] and other types of solid cancers.

Author Contributions

EF and DF acquired data; EF, DF, AP, CP and JAF analysed and interpreted data; RF and MRS produced the artwork; EF, DF, AP, GM, LLS and JAF drafted the article and critically revised it; EF, DF, AP, AB, LLS and JAF approved the final version of the draft to be submitted. JAF and BS conceptualized and design the study.

Conflict of interests

The authors declare having no conflict of interests.

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Glycoengineered nanoparticles enhance the delivery of 5-fluorouracil and paclitaxel to gastric cancer cells of high metastatic potential

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SUPPLEMENTARY MATERIAL

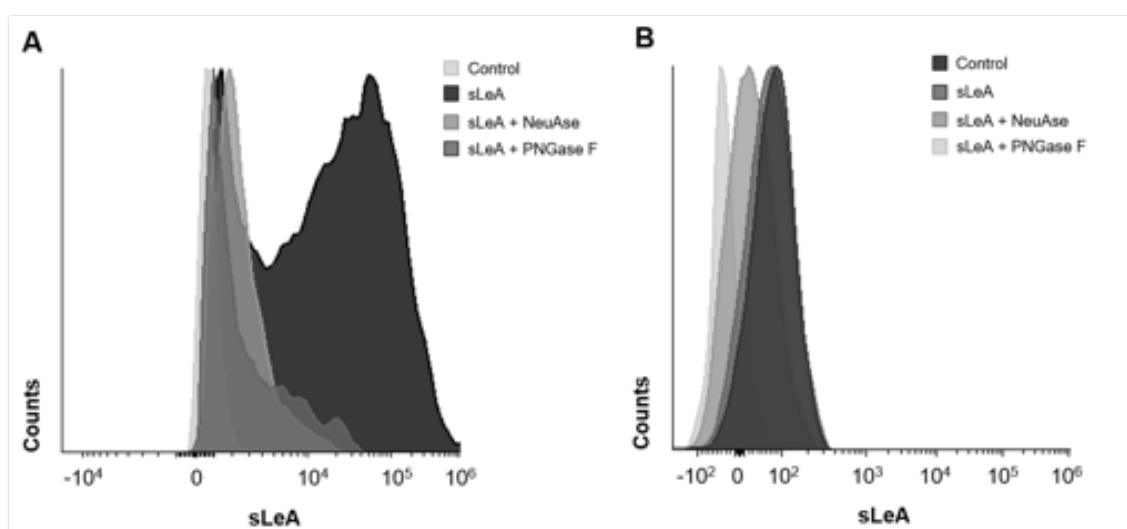


Figure S1. Flow cytometry analysis of gastric cancer cell lines AGS and NCI-N-87. Autofluorescence, secondary antibody and isotype negative controls were set. Enzymatic controls using α -Neuraminidase and PNGase F were also included to disclose signal specificity and *N*-glycan contribution for SLe^A cell surface expression, respectively. Accordingly, the metastatic gastric cancer cell line overexpresses SLe^A, while there is no significant expression of the antigen in AGS cells. Two independent experiments were performed in triplicate for each cell line and condition. At least 20 000 events were acquired for each condition.

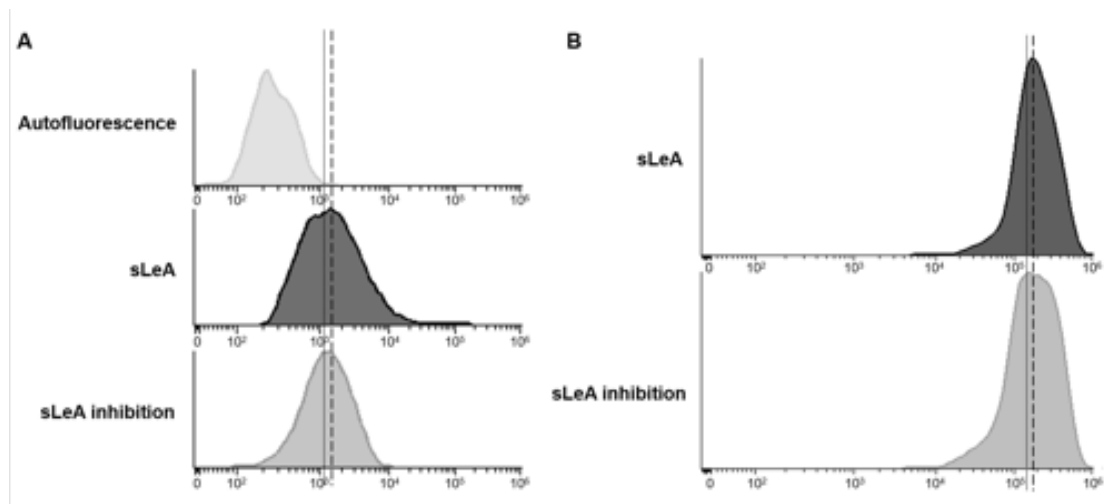


Figure 2S. Flow cytometry analysis of NCI-N-87 cells upon 2FF treatment. NCI-N-87 cells were incubated with 2FF, a known inhibitor of fucosyltransferases responsible by SLe^A biosynthesis. SLe^A inhibition resulted in a decrease in the total amount of cell surface antigen (**A**) and a decrease in NPs internalization (**B**) in relation to the median point.

CHAPTER 3

Glycomics and Glycoproteomics characterization of gastric cancer cell models identify SLe^A-Nucleolin glycoforms

The information provided in this chapter presents part of unpublished data of a manuscript in preparation by Elisabete Fernandes et al.

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Glycomics and Glycoproteomics characterization of gastric cancer cell models identify Sialyl-Lewis A-Nucleolin glycoforms as potentially targetable biomarkers at the cell membrane

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Abstract

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide, with half of patients developing metastasis within 5 years after treatment. Moreover, the introduction of targeted therapeutics has been challenged by high intra- and inter-tumoral molecular heterogeneity and limited cancer biomarkers specificity. Exploiting the sialyl-lewis A (SLe^A) antigen, a terminal glycoepitope of membrane glycoproteins and glycolipids, offers tremendous potential towards more precise cancer targeting. This glycan is rarely expressed by healthy tissues and normal blood cells but is often present in cancer cells of high metastatic potential as well as in cancer metastasis. The SLe^A antigen is also directly involved in E-selectin mediated metastasis, making it an ideal target to control disease dissemination. To improve further on the cancer nature of this glycan we have zoomed in on the SLe^A-glycoproteome envisaging targetable biomarkers. An initial glycomics screening of well-known cell models reflecting different histological subtypes of the disease identified significant SLe^A levels in KATO-III, NCI-N87 and OCUM-1 cells in both *N*- and *O*-glycoproteins, which was later confirmed by O-glycomics. SLe^A-expressing glycoproteins were then isolated from these cells by immunoprecipitation and identified by nanoLC-ESI-MS/MS. Glycoproteins with affinity for E-selectin were also identified. Marked differences in glycoproteomics signatures were observed between cell lines, reinforcing expected molecular heterogeneity. However, a restricted panel of SLe^A-expressing glycoproteins with affinity for E-selectin were identified in all cell lines. An in-house bioinformatics-assisted algorithm identified nucleolin (NCL), a nuclear protein frequently observed at the cell-surface in cancer cells, as a potential targetable biomarker. Western blot, flow cytometry and immunofluorescence studies reinforced the existence of SLe^A-NCL glycoforms at the cancer cells surface and in gastric tumours, supporting mass spectrometry findings. Moreover, the SLe^A-NCL glycophenotype was not reflected in relevant healthy tissues, supporting its tremendous potential envisaging precise cancer targeting with limited off-target effects.

Collectively, we have demonstrated that the miss-localization of nuclear NCL to the cell surface may be used to selectively target cancer cells presenting higher metastatic potential translated by higher affinity to E-selectin. To our knowledge, this is the first report describing SLe^A in a protein preferentially found in the nucleus, setting a new paradigm for cancer biomarker discovery.

Introduction

Gastric cancer(GC) is the third leading cause of cancer-related death worldwide and constitutes a significant therapeutic hurdle due to the lack of efficient therapeutics to address disseminated disease [1, 2]. As such, half of patients develop metastasis within 5 years after curative treatment comprehending surgery, chemotherapy and/or radiotherapy [3, 4]. Moreover, many patients cannot tolerate or complete systemic treatment due to severe treatment side-effects [5, 6]. The introduction of antibody-based targeted therapeutics with trastuzumab (anti-HER2), ramucirumab (anti-VEGFR2), and cetuximab (anti-EGFR) has provided only modest improvements in patient survival and frequently fail to control metastatic spread [7-10] . In addition, these options serve a reduced number of patient's subpopulations and lack the necessary tumour specificity to fully avoid off-target related toxicity [11]. These clinical challenges centre the spotlight on a novel quest for cancer-specific biomarkers capable of overcoming significant inter- and intra-tumoral molecular heterogeneity.

Malignant transformation is accompanied by a profound remodeling of the plasma membrane glycome and glycoproteome, generating cancer unique molecular fingerprints for precise cancer targeting [12, 13]. Premature stop in *O*-glycan (in Ser and Thr residues) extension in membrane bound proteins as well as oversialylation and fucosylation of distinct glycoepitopes are amongst the most well-known events [13, 14]. Namely, gastric tumours often overexpress sialylated Lewis (SLe) antigens such as sialyl-Lewis A (SLe^A) and its isomer sialyl-Lewis X (SLe^X), which are terminal epitopes of glycan chains in proteins and lipids present at the cell-surface [15]. However, gastric tumours have been found to significantly express

SLe^A, showing only modest increases of SLe^x levels [16]. It is also well documented that these sialylated Lewis antigens mediate binding to E-selectin, promoting tumour cells arrest in blood vessels, extravasation into the bloodstream and homing to distant organs [12, 17]. In fact, the interaction between SLe-expressing cancer cells and E-selectin, a calcium-dependent lectin overexpressed by microvascular endothelial cells during inflammation, is pivotal for cancer spread [18, 19] and its inhibition has significantly improved survival in animal cancer models [20]. Accordingly, GC patients with tumours showing pronounced SLe^A expression have been found at greater risk of developing distant metastasis and, consequently, face worst prognosis [21]. This antigen has also been observed in GC metastasis [22], suggesting potential for designing more effective therapeutics. Even though the SLe^A is not expressed by healthy blood cells, low amounts of this glycoepitope may be found in the salivary glands, colon, pancreas, bile ducts and other healthy tissues [16, 23]. It has also been observed in several non-malignant conditions of the gastrointestinal tract limiting its biomarker potential [24-26]. Such observations challenge the cancer-specificity of the glycoantigen, urging the identification of protein glycoforms for precise targeting. As such, this work comprehensively interrogates the glycome and glycoproteome of wide panel of GC cell models as a starting point for the identification of unique SLe^A molecular signatures. Emphasis was set on SLe^A protein glycoforms with affinity for E-selectin and limited expression in healthy tissues. We anticipate that this may constitute an important roadmap towards improved disease management at different levels.

Material and Methods

Cell lines and culture conditions

Human GC cell lines OCUM-1 and MKN-74 were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, whereas NCI-N87, AGS, KATO-III and MKN-45 cells were acquired from ATCC. Cells were maintained with 10% heat-inactivated FBS (Gibco, Life Technologies) and 1%

penicillin-streptomycin (10,000 Units/mL penicillin; 10 000 mg/mL streptomycin; Gibco, Life Technologies) supplemented RPMI 1640+GlutaMAX™-I medium (Gibco, Life Technologies). Exceptionally, OCUM-1 cells were grown with Low glucose DMEM (GE Healthcare Life Sciences). Cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Gastric tumours and healthy tissues

The screening of SLe^A and NCL was performed on 50 formalin-fixed paraffin embedded (FFPE) tissue sections collected from surgical patients in the Portuguese Institute of Oncology of Porto (IPO-Porto, Portugal) between 2004 and 2016. The tissue sections were collected from 25 male and 27 females with age between 34 and 89 years. All clinicopathological information was obtained from patient's clinical records. A broad library of healthy tissues (appendix, skin, lung, pancreas, colon, small intestine, gallbladder, liver, testis, stomach, thyroid and tonsil) were also screened for these two markers. All procedures concerning the inclusion of patients were approved by the institutional Ethics Committee after patient's informed consent.

Flow cytometry

Cells were detached using Versene solution (ThermoFisher, Waltham, MA, USA), fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and stained with mouse anti-SLe^A monoclonal antibody [CA19.9-9-203] (ab116024, Abcam) using a 1:100 dilution in PBS 2% FBS for 1 hour at room temperature. Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 488 was used for SLe^A detection at a 1:300 dilution in PBS 2% FBS for 15 min at room temperature. Mouse IgG1 [MOPC-21] isotype control (ab18443, Abcam) was included as a negative control. In parallel, 10⁶ cells were digested with either 10 mU/mL Neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) or 25mU/mL PNGase F from *Elizabethkingia meningoseptica* (Sigma-Aldrich) at 37°C for 1 hour under mild agitation,

prior to SLe^A staining. PNGase F digestion assay was used to access the overall SLe^A expression in glycoprotein *N*-glycans, whereas Neuraminidase treated cells were included as negative controls. In addition, cancer cells were screened for NCL expression using the Alexa Flour 647-labelled rabbit anti-human NCL monoclonal antibody [EPR7952] (ab202709, Abcam). NCL was evaluated using membrane intact live cells in the presence of propidium iodide (PI, Thermo Fisher Scientific) to reassure cell surface antigen detection. Control experiments included cells permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) prior to FACS analysis to determine the total NCL content. Data analysis was performed through CXP Software and results represent the standard deviation of three independent experiments.

Subcellular protein fractioning

Cells were first lysed using a 20mM HEPES, 10mM KCl, 2mM MgCl₂, 1mM EDTA and 1mM EGTA buffer and passed through a 27-gauge needle. Subcellular fractions (nuclei, cytoplasm and membrane) were obtained by differential centrifugation at 720*g* (for nuclei precipitation) and 100,000*g* (for plasma membrane proteins precipitation). The pellets were then resuspended in TBS with 0.1% SDS and the amount of protein was estimated using a DC protein assay kit (BioRad).

O-glycomics

Gastric cancer cell models *O*-glycome was characterized using Cellular *O*-glycome Reporter/Amplification method, as previously described [27]. Briefly, benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside (Sigma-Aldrich) was peracetylated and administered to semi-confluent cells to a final concentration of 150 μ M. Following 24h incubation, glycans were isolated from the conditioned media by filtration using 10 kDa centrifugal filter (Amicon Ultra-4, Merck Millipore) followed by reversed-phase chromatography in Sep-Pak 3 cc C18 cartridges (Waters). Finally, Bn-*O*-glycans were permethylated and analyzed by nanoLC-ESI-MS/MS using a nanoLC system (Dionex, 3000 Ultimate nano-LC) coupled online to an LTQ-

Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source (Thermo Scientific, EASY-Spray source). Eluent A was aqueous formic acid (0.2%) and eluent B was formic acid (0.2%) in acetonitrile. Samples (20 μ l) were injected directly into a trapping column (C18 PepMap 100, 5 μ m particle size) and washed with an isocratic flux of 95% eluent A and 5% eluent B at a flow rate of 30 μ l/min. After 3 minutes, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 $\text{\AA}$, 150 mm $\times$ 75 μ m ID and 3 μ m particle size) at a flow rate of 0.3 μ l/min. Column temperature was set at 35°C. Permethyated glycan separation occurred using a linear gradient of 10% eluent B over 10 min, 10-38% eluent B over 10 min, 38-50% eluent B over 35 min, 50-90% eluent B over 10 min and 90% eluent B over 10 min. The mass spectrometer was operated in the positive ion mode, with a spray voltage of 1.9 kV and a transfer capillary temperature of 250°C. Tube lens voltage was set to 120 V. MS survey scans were acquired at an Orbitrap resolution of 60,000 for an m/z range from 300 to 2000. Tandem MS (MS/MS) data was acquired in the linear ion trap using a data dependent method with dynamic exclusion. The top 6 most intense ions were selected for collision induced dissociation (CID). CID settings were 35% normalized collision energy, 2 Da isolation window 30 ms activation time and an activation Q of 0.250. A window of 90 s was used for dynamic exclusion. Automatic Gain Control (AGC) was enabled and target values were 1.00e+6 for the Orbitrap and 1.00e+4 for LTQ MS analysis. Data were recorded with Xcalibur software version 2.1. Glycan structures were assigned based on characteristic product ion spectra and previous knowledge about O-glycosylation pathways [27]. Glycans were expressed in terms of relative abundance in comparison to the sum of all individual contributions to the glycome. Triplicate experiments were performed.

Glycoproteomics

The SLe^A expressing glycoproteins were isolated from plasma membrane enriched extracts (200 μ g) by immunoprecipitation using the anti-SLe^A monoclonal antibody [CA19.9-9-203] (ab116024, Abcam) immobilized at the surface of magnetic beads with the Pierce™ Direct IP Kit (26148, ThermoFisher Scientific), according to the manufacturer's instructions. In parallel, a similar strategy was used to pull-down glycoproteins with affinity for E-selectin [28]. A recombinant mouse E-selectin/ human Fc chimera (E-selectin-Ig chimera-E-Ig), a validated tool to identify E-selectin ligands in human cells [29, 30], was used towards this end. The E-Ig chimera was immobilized at the surface of magnetic beads, as previously described, and incubated with the membrane protein extracts containing 2mM CaCl₂. The glycoproteins isolated in these assays were then resolved by SDS-PAGE using 4–20% precast polyacrylamide gels (Bio-Rad). The bands were excised from the gels, reduced with DTT (Sigma), alkylated with iodoacetamide (Sigma) and incubated with trypsin (Promega), as previously described [31, 32]. The resulting peptides were analyzed by nanoLC-ESI-MS in a nanoLC system (Dionex, 3000 Ultimate nano-LC) coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source (Thermo Scientific, EASY-Spray source), according to the conditions previously described by Cotton et al. [31]. Data was analyzed automatically using the SequestHT search engine with the Percolator algorithm for validation of protein identifications (Proteome Discoverer 1.4, Thermo Scientific). Data was searched against the human proteome obtained from the SwissProt database on 22/11/2015, selecting trypsin as the enzyme and allowing up to 2 missed cleavage sites and a precursor ion mass tolerance of 10 ppm and 0.6 Da for product ions. Carbamidomethylcysteine was selected as a fixed modification, while oxidation of methionine (+15.9) and modifications of serine and threonine with HexNAc (+203.079; Tn antigen), HexNAc-NeuAc (+494.175; sialyl-Tn antigen); HexNAc-Hex (+365.132; T antigen), HexNAc-Hex-NeuAc (+656.228, sialyl-T antigen); HexNac(NeuAc)-Hex-NeuAc (+947.323, disialyl-

T antigen), HexNAc(Hex)-HexNAc(Fuc)-Hex(NeuAc) (+ 1167.418, core 2 substituted with a terminal SLe^A epitope) were defined as variable modifications, based on glycomics studies. Protein grouping filters were set to consider peptide-to-spectrum matches (PSMs) with low confidence and ΔC_n better than 0.05 to accommodate limitations associated with glycopeptides annotation based on CID fragmentation [31, 33]. At least two PSMs per protein were required and the strict maximum parsimony principle was applied for positive protein annotation. Accordingly, the glycopeptide annotations list included high and medium/low confidence identifications. For definitive confirmation, all low scoring glycopeptides were subjected to additional manual confirmation backed by GlycoPAT, a comprehensive open-source platform for MS-based glycoproteomics data analysis [34]. The results corresponded to proteins identified with high confidence and showing glycopeptides with SLe^A as post-translational modification (PTM).

Bioinformatics for biomarker discovery

The subcellular location and molecular and biological functions associated with the subsets of sLe^A-expressing glycoproteins with affinity for E-selectin for each cell line were characterized using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 10.5 (<http://string-db.org/>) [35]. The glycoproteins common to all the cell lines were also categorized based on transcriptomics data deposited in OncomineTM (<https://www.oncomine.org/>) [36] for distinct GC subtypes (intestinal, mixed, diffuse) compared to the healthy gastric mucosa. A $p \leq 0.05$ as well as a 2-fold variation of expression were considered. This data was integrated with information on protein expression in the normal gastric mucosa from The Human Protein Atlas [37, 38]. Protein expression data deposited in the Human Protein Atlas was further used to develop a scoring algorithm (target score) to rank the identified glycoproteins in relation to their potential for targeted therapeutics with minimal off-target effects. To achieve this goal, the target score majorated the overexpression and localization of the glycoproteins at the cell membrane as well as associations

with poor prognosis in GC, while penalizing their presence in the same subcellular compartment as in healthy tissues. Accordingly, the score system results from the sum of the following nine variables: i) location in healthy cells (membrane: 0 points; other subcellular locations: 1 point); ii) location in cancer cells (membrane: 1 point; membrane and/or other subcellular locations: 0 points); iii) expression in the healthy gastric epithelium at the plasma membrane (negative: 3 points; low: 2 points; moderate: 1 point; high: 0 points); iv) expression in GC (negative: 0 points; low: 1 point; moderate: 2 points; high: 3 points); v) prognosis value in GC (high expression is not prognostic and/or associates with favorable prognosis: 0 points; high expression associates with poor prognosis: 1 point); vi) expression in the brain (exclusively in the brain: 1 point; brain and other tissues: 0 points); vii) expression in lymphoid tissues at the plasma membrane (not expressed: 1 point; expressed: 0 points); viii) expression in gametes (not expressed: 1 point; expressed: 0 points); ix) index of expression in healthy tissues at the plasma membrane-varies from 3 points (no expression) to 0 (high expression). Interpretation of biological functions associated to glycoproteins common to all cell lines and showing affinity to E-selectin was done using ClueGO version 2.2.5 [39] and CluePedia plugins version 1.2.5 [40] for cytoscape version 3.3.0 [41].

Western blot

Proteins from different sources were resolved by SDS-PAGE using 4–20% precast polyacrylamide gels (Bio-Rad) and then transferred to a nitrocellulose membrane (GE Healthcare Life Sciences). This included total protein extracts and extracts for proteins from different subcellular fractions (plasma membrane, nucleus, cytoplasm). It also included NCL and SLe^A-expressing glycoproteins isolated by immunoprecipitation using an anti-SLe^A monoclonal antibody (ab116024, Abcam) or anti-NCL antibody [EPR7952] (ab129200, Abcam), as described in the glycoproteomics section. The membranes were then blocked with 1% Carbo-Free Blocking Solution (Vector Laboratories) and probed with either the anti-SLe^A (1:1000) or the anti-NCL (1:1000) antibodies described above for 1 hour at room

temperature. The peroxidase affiniPure goat anti-mouse IgG (H+L) polyclonal antibody (1:90 000, 115-035-003, Jackson ImmunoResearch) was used as a secondary antibody for SLe^A detection. The goat anti-rabbit IgG (H+L) HPR conjugate antibody (1: 60 000; G-21234; ThermoFisher Scientific) was used as a secondary antibody for NCL detection. Additional enzymatic controls were performed prior to blotting for SLe^A. Briefly, membranes were incubated with 0.2U Neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) or 0.16U/mL PNGase F from *Elizabethkingia meningoseptica* (Sigma-Aldrich) overnight at 37°C to disclose the specificity of SLe^A expression. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent

Immunofluorescence and *in situ* proximity ligation assays (PLA)

Cells were cultured in coverslips and fixed with 4% PFA. After blocking with 5% bovine serum albumin (BSA), cells were stained with the anti-SLe^A and anti-NCL primary antibodies as above described. Enzymatic controls with neuraminidase from *Clostridium perfringens* or PNGase F from *Elizabethkingia meningoseptica* and permeabilization with 0.1% Triton X-100 (Sigma-Aldrich) were included. The *in-situ* proximity ligation assay (PLA) of SLe^A and NCL in cell lines was performed to detect both antigens in simultaneous proximity. The slides were incubated overnight with the primary antibodies at 4°C. Ligation and amplification of the PLA signal were performed according to the Duolink PLA Technology kit (Sigma-Aldrich). Finally, slides were incubated with DAPI and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). All images were acquired on a Leica DMI6000 FFW microscope and Las X software (Leica).

Immunohistochemistry

Formalin-fixed paraffin embedded (FFPE) GC tissue sections showing distinct clinicopathological features (intestinal and diffuse type GC) and healthy tissues (appendix, skin, lung, pancreas, colon, small intestine,

gallbladder, liver, testis, stomach, thyroid and tonsil) were screened by immunohistochemistry for both SLe^A and NCL [31], as previously described by us. Briefly, FFPE tissue sections were deparaffinized with xylene, rehydrated with a graded series of alcohol washes and subjected to heat-induced antigen retrieval using citrate buffer pH=6.0 (Vector Laboratories) for 15 min in the microwave, after pre-heating of the solution at maximum power rating for 5 min. Sections were incubated with 0.3% hydrogen peroxide (Merck KGaA) for 25 min, blocked with UV Block® (Thermo Scientific, TL-060-HD) and incubated overnight at 4 °C in a wet chamber with either anti-SLe^A (1:100 5% BSA-PBS) or anti-NCL (1:250 5% BSA-PBS) antibody. After washing with PBS-Tween, biotinylated secondary antibody was added to tissue sections, before incubation with streptavidin (Thermo Scientific, 434301). Antibodies' binding was detected by incubation with 3,3'-diaminobenzidine (ImmPACT™ DAB, Vector Laboratories, SK-4105) for 4 min. Nuclei were counterstained with hematoxylin for 1 min. Positive and negative control sections were tested in parallel. Negative control sections were performed using 5% BSA-PBS devoid of primary antibody. Positive controls consisted of known positive tumour tissues for the antigen in study. Tumours were classified as positive when immunoreactivity was observed by microscopic presence of brown chromogenic product in tumour cells. Antibodies' staining was assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. To evaluate sialic acid-dependent binding of the antibodies, tissues were treated with sialidase prior to analysis.

Statistical Analysis

One-way ANOVA and Non-parametric T-tests were used. Differences were significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The obtained data were analyzed by GraphPad Prism 6 (GraphPad Software).

Results and Discussion

The expression of SLe^A at the cell-surface is decisive for E-selectin mediated hematogenous dissemination of GC cells and the formation of local and distant metastasis [15]. However, the nature of the glycoprotein carrying this modification in GC cells remains to be elucidated, which is a critical aspect for precise targeting of subpopulations showing high metastatic potential. As such, this work started by screening a panel of cell models reflecting distinct aspects of the disease for SLe^A. The overall goal was to elect models for downstream targeted glycoproteomics studies foreseeing the identification of SLe^A-expressing glycoproteins showing high affinity for E-selectin. Emphasis was set on disclosing alterations in the *O*-glycoproteome, that is well known to experience significant remodeling during GC progression and dissemination [14, 42].

Glycomics

Six cancer cell models were screened for SLe^A by flow cytometry. FACS analysis (**Figure 1 A and B**) showed that AGS and MNK-74 cells did not express SLe^A. On the other hand, high levels of this glycan were observed in N87 and OCUM-1 (60-80% of positive cells) and, in much less extent, in MKN-45 and KATO III cells (>20%). Cells were subsequently *N*-deglycosylated with PNGase F to determine the origin of the SLe^A antigen. This had little impact in MKN-45, KATO III and OCUM-1, strongly suggesting that SLe^A may mostly derive from *O*-glycans in glycoproteins and, potentially, glycolipids (**Figure 1 A-C**). On the other hand, exposure to PNGase F significantly decreased SLe^A levels in N87 cells (4-fold), demonstrating that most of the antigen comes from glycoprotein *N*-glycans. Nevertheless, 20% of cells retained SLe^A expression after enzymatic treatment, also suggesting significant *O*-glycosylation levels. Notably, the signals in all cell lines were highly responsive to neuraminidase digestion, demonstrating the specificity of the anti-SLe^A antibody for sialylated glycans (**Figure 1 A and B**).

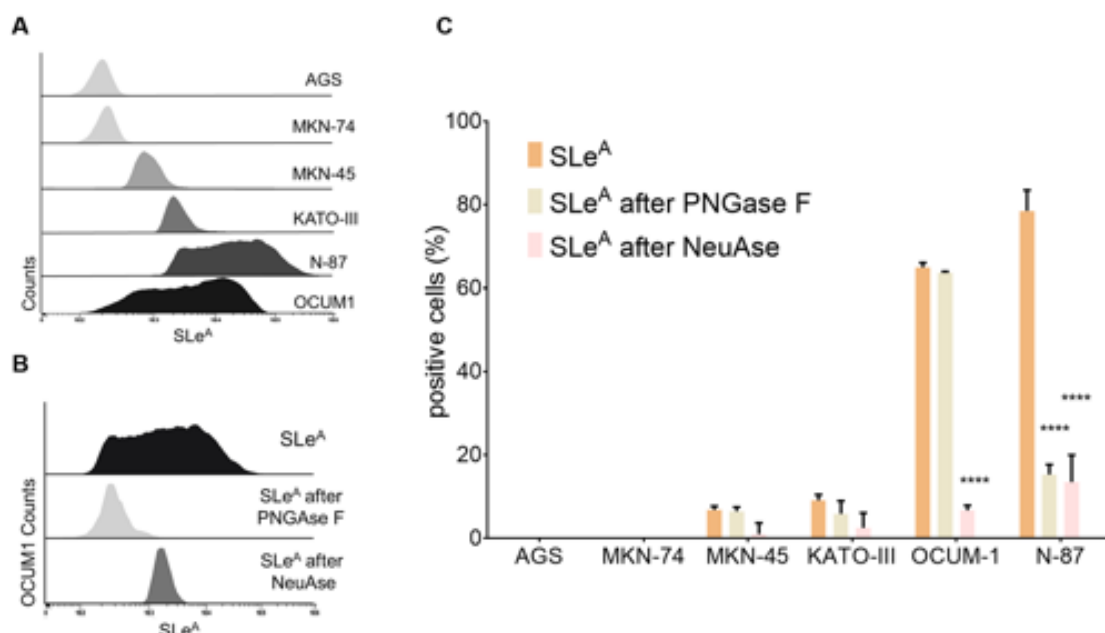


Figure 1. Expression of SLe^A in different GC cell models. The FACS in panel A highlights the levels of SLe^A in AGS, MKN-74, MKN-45, KATO-III, N87 and OCUM-1 cells, ranked from the lowest to highest expression. Notably, AGS and MKN-74 were negative for the antigen, whereas N87 and OCUM-1 presented the highest expressions. The FACS in panel B correspond to control experiments involving PNGase F (for *N*-deglycosylation) and neuraminidase (for removal of sialic acids) for cell line N87. This confirmed the specificity of the anti-SLe^A antibody for sialylated structures and that the SLe^A signals arised mostly from *N*-glycans. However, a still significant subpopulation of cells conserved SLe^A antigen expression, suggesting also the presence of *O*-glycans and/or glycolipids carrying this alteration. Panel C summarizes SLe^A evaluation for each cell line resulting from FACS experiments, in agreement with the observations in panel A. Exposure to neuraminidase had little effect on MKN-45, KATO-III and OCUM-1 cell lines, suggesting that the majority of the SLe^A may arise from the *O*-glycosylation of proteins and also from glycolipids. In N-87 the major source of SLe^A expression appear to be *N*-glycans. Nevertheless, considerable amounts of SLe^A remain suggesting also the presence of *O*-glycans. Moreover, these signals were significantly decrease when the cells were treated with neuraminidase prior to analysis, reinforcing the specificity of antibody recognition. “*” $p < 0.05$; “**” $p < 0.01$; “***” $p < 0.001$; and “****” $p < 0.0001$. (Student’s T-test for 3 independent replicates)

We then comprehensively interrogated the cells glycome by mass spectrometry with the objective of validating these results. This also provided more insights on the structure of the glycans exhibiting this terminal epitope, which was also of key importance for protein annotation upon glycoproteomics characterization. Based on flow cytometry analysis

and previous reports, emphasis was set on the *O*-glycome [42]. As highlighted by **Figure 2A**, 23 distinct glycans were identified. Overall, the glycome of all cell lines was composed by core 1 and core 2 fucosylated and/or sialylated *O*-glycans of variable lengths. Nevertheless, shorter glycans predominated over more extended structures beyond core 2 (**Figure 2A and B**) and all cell lines, irrespectively of their origin, overexpressed the T (m/z 572.3), fucosyl-T (m/z 746.4), ST (m/z 933.5) and short core 2 (m/z 1021.5; 1195.6) antigens. However, key differences could be identified when zooming in on the glycome of each cell line. According to the heat map on **Figure 2B**, OCUM-1, AGS and KATO-III cell lines were enriched for neutral glycans, including core 1 (T antigen; m/z 572.3), fucosylated core 1 (fucosylated T antigen; m/z 746.3) and shorter core 2 structures (m/z 1021.5; 1195.6), exhibiting considerable degrees of mono- (m/z 1225.6), di- (m/z 1369.7; 1831.9) and tri-fucosylation (m/z 1543.8). However, OCUM-1 mainly overexpressed shorter core 1 glycans, whereas AGS and KATO-III presented higher relative amounts of core 2 structures. Glycomics analysis also suggested that the levels of fucosylation were higher in AGS in comparison to KATO-III cells. The pronounced neutral character presented by OCUM-1, AGS and KATO-III glycomes (*e.g.* m/z 572.3, 746.4, 1195.6) clearly contrasted with the oversialylated phenotype of MKN-45, MKN-74 and N87 (*e.g.* m/z 933.5, 1178.7, 1294.7) cells. Nevertheless, MKN-45 exhibited a higher amount of more extended fucosylated and/or sialylated glycans, MKN-74 were significantly enriched for the sialyl-T antigen (ST antigen; m/z 933.5) and N87 cells presented shorter mono- and di-sialylated core 1 and core 2 glycans, including sialyl-Tn (STn antigen; m/z 729.4), a glycan known to play a critical role in GC invasion and metastasis [43, 44]. Despite marked differences, MKN-45, KATO-III, OCUM-1 and N87 also exhibited low abundant ions (<5% relative abundance; m/z 1556.9, 1801.9 and 1917.9) potentially corresponding to glycans with terminal SLe^A epitopes, supporting flow cytometry analysis, which was confirmed by tandem mass spectrometry (**Figure 2C**). In summary, glycomics characterization provided important structural information for guiding future studies on the functional role of *O*-glycans in GC. Moreover, it demonstrated that the SLe^A is not a major

determinant of the *O*-glycome, despite its tremendous impact on metastasis development. It was also crucial in the identification of glycan modifications to be used in targeted glycoproteomics, enabling higher protein coverage and the validation of relevant glycosylated patterns.

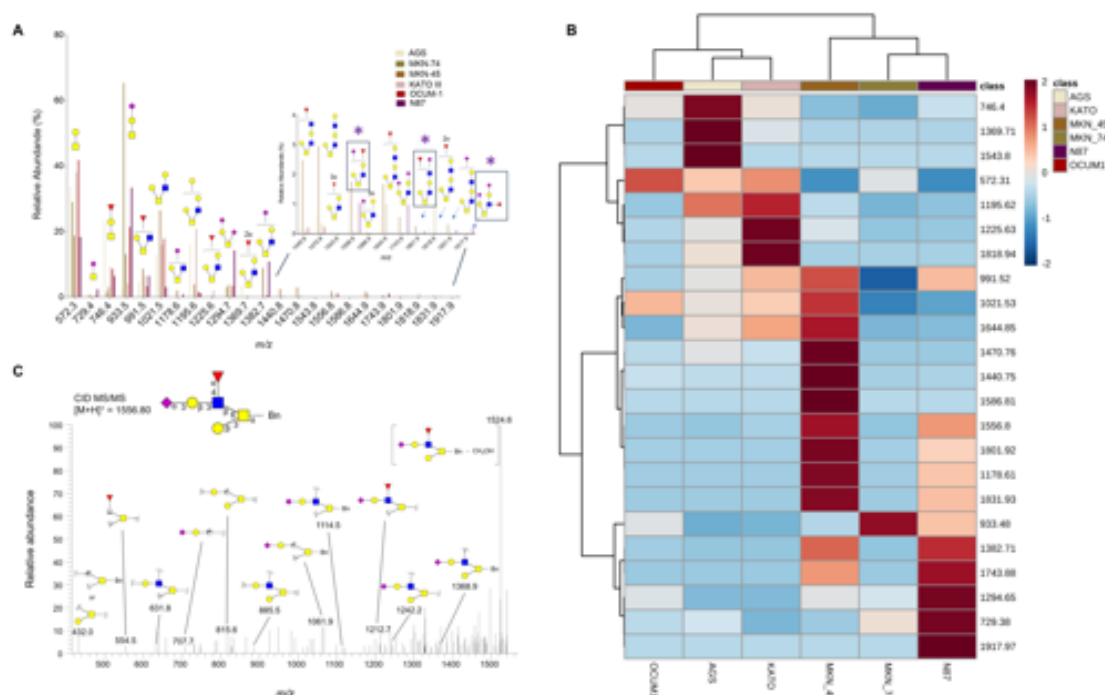


Figure 2. *O*-Glycomics analysis of GC cells. Panel A shows that the *O*-glycome of all cell lines was composed by core 1 and core 2 fucosylated and/or sialylated *O*-glycans of variable lengths. Also, shorter glycans predominated over more extended structures beyond core 2. All cell lines, irrespectively of their origin, overexpressed the T (m/z 572.3), fucosyl-T (m/z 746.4), ST (m/z 933.5) and short core 2 (m/z 1021.5; 1195.6) antigens has main *O*-glycans. More detailed differences and similarities presented by these models are highlighted by the heatmap and cluster analysis in Panel B. Notably, cell lines MKN-45, KATO-III, OCUM-1 and N87 also exhibited low abundant ions (<5% relative abundance; m/z 1556.9, 1801.9 and 1917.9) potentially corresponding to glycans with terminal SLe^A epitopes, supporting flow cytometry analysis. This hypothesis was confirmed by tandem mass spectrometry in Panel C, which also showed SLe^A diagnostic ions.

Targeted glycoproteomics and glycobiomarker discovery

We then devoted to characterize the SLe^A glycoproteome of different cancer cell models with the goal of identifying common molecular signatures for precise cancer targeting. OCUM-1, N87 and KATO-III cell lines showing relevant SLe^A levels were elected for this study. Glycoproteins were isolated from extracts enriched for plasma membrane proteins by immunoprecipitation with an anti-SLe^A monoclonal antibody. These were subsequently resolved by SDS-PAGE and identified using a conventional bottom-up proteomics strategy (excision of gels spots, reduction, alkylation, and digestion with trypsin) culminating in nanoLC-ESI-MS/MS analysis. In parallel, glycoproteins with affinity for E-selectin were also pulled-down with an E-selectin-IgG chimera and identified as described above to disclose potential mediators of metastasis development. Protein annotations were performed including the possibility of variable protein modifications with the Tn antigen (the simplest form of *O*-glycosylation) as well as the T, ST, fucosylated-T and extended core 2 substituted with sialylated Lewis antigens, in accordance with glycomics analysis. The final list of identifications included proteins annotated with high confidence, irrespectively of the SLe^A status.

Immunoprecipitation assays resulted in identification of 889, 344 and 694 proteins in N87, OCUM-1 and KATO-III cell lines respectively, demonstrating that the number of protein annotations was not directly linked to SLe^A levels (according to flow cytometry). Interestingly, few glycoproteins (less than 10%) were common to all cell lines, denoting highly cell-specific SLe^A-glycoproteomes (**Figure 3**). However, the number of glycoproteins showing affinity for E-selectin was lower in comparison to SLe^A-immunoprecipitation assays (630 proteins for N87, 309 for OCUM-1 and 579 for KATO-III). Nevertheless, on average, 60% of the identified glycoproteins were simultaneously identified by the two strategies for each cell line (532 for N87, 186 for OCUM-1, 440 for KATO-III). Moreover, MS/MS analysis allowed to confirm the presence of *O*-glycopeptides with SLe^A epitopes in approximately 30% of the identified glycoproteins in each cell

line, confirming glycomics studies and protein identifications by mass spectrometry. However, we have also noted that a significant percentage of the glycoproteins isolated by SLe^A IP did not show affinity to E-selectin (approximately 40% for all cell lines). Even though the existence of non-specific immunoprecipitations is frequent and may decisively contribute to these observations, approximately 50% of the unmatched glycoproteins in the SLe^A-IP group exhibited SLe^A-glycopeptides. Such observations strongly suggest that the expression of sialylated Lewis antigens is not a decisive pre-requisite for E-selectin binding. It is possible that the nature of the glycoproteins presenting these modifications as well as the density and structural organization of the glycans chains may also influence this process, which warrants a more profound evaluation in future studies. On the other hand, there is also a significant portion of E-selectin identifications that do not find match in the SLe^A-IP group. The expression of SLe^x by these cell lines, which is also a key ligand for E-selectin [15], may also account for these findings. In summary, we have demonstrated the existence of a wide array of glycoproteins showing high affinity for E-selectin, including many glycoproteins with confirmed *O*-SLe^A glycoforms (193 glycoproteins). These were distributed amongst the three cell lines according to the Venn Diagram in **Figure 3D**. The N87 model presented a more unique glycopattern in comparison to the other cell lines (95 unique glycoproteins in N97 vs 4 for OCUM-1 and 1 for KATO-III). A high degree of homology was also observed between N87 and KATO-III cell lines at this level (55 common glycoproteins). More importantly, 22 protein species were common to all cell lines (corresponding to 12% of the total identifications), suggesting a relatively low degree of similarity between the three cell models. Nevertheless, the identified glycoproteins constituted an important starting point for identification of common molecular grounds potentially associated to metastasis development.

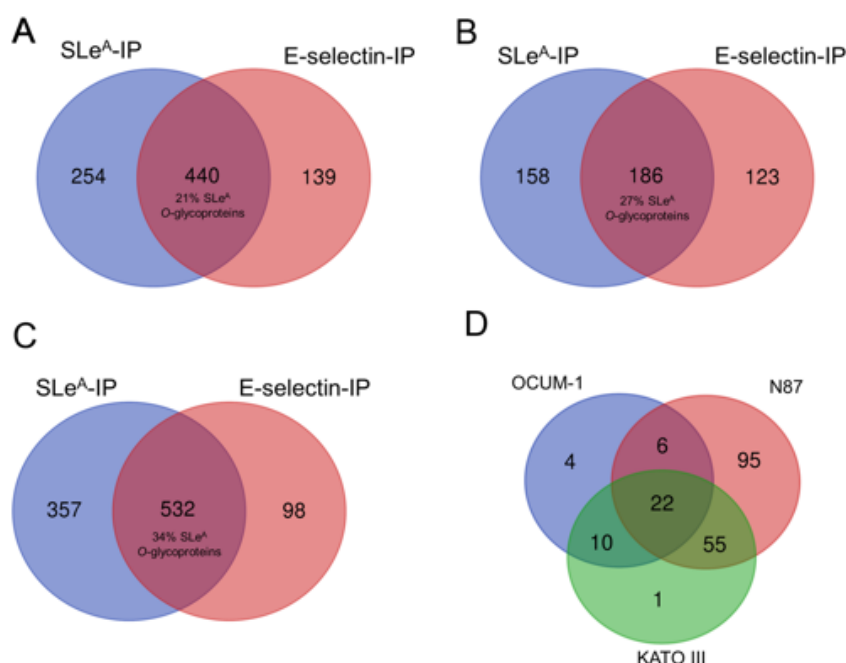


Figure 3. Venn diagrams highlighting the distribution SLe^A-expressing glycoproteins and glycoproteins showing affinity for human E-selectin for cell line KATO-III (A), OCUM-1 (B), N87 (C) and glycoproteins with confirmed O-SLe^A expressing glycosites and showing affinity for E-selectin amongst the three cell models (D). The Venn Diagrams in panels A-C highlight the number of glycoproteins isolated by IP for SLe^A and E-selectin for each cell lines, highlighting the percentage of glycoproteins commonly identified for both IPs and showing clear SLe^A modifications in O-glycans. Panel C highlights that only a restricted number of 22 glycoproteins presenting O-SLe^A glycosylation and affinity for E-selectin could be found in all cell lines.

Envisaging molecular signatures of potential clinical interest for a wide number of tumours, focus was set on the restricted group of 22 glycoproteins found in all cell lines. According to transcriptomics data deposited on the Oncomine repository, only 8 glycoproteins were significantly overexpressed in GC (*KRT5*, *HIST1H1D*, *PSMD2*, *SPTBN1*, *MYOF*, *AHNAK2*, *ANX2* and *SPTAN1*), distributed between the different types of gastric tumours according to the Lauren classification (intestinal, diffuse and mixed type adenocarcinomas), as highlighted in **Figure 4A**. Gene expression was then matched with the levels of the proteins in gastric tumours, as defined by the Human Protein Atlas (**Figure 4B**). Notably, *HIST1H1D*, *PSMD2*, *SPTBN1*, *MYOF*, *ANXA2* and *SPTAN1* overexpression was associated with elevated protein content in cancer tissues whereas *KRT5* has

been rarely observed at the protein level in cancer tissues (**Figure 4B**). Contrastingly, HIST1H1D overexpression was observed in all three types of gastric lesions, PSMD2 and SPTBN1 was mainly characteristic of intestinal and mixed type tumours, whereas MYOF and AHNK2 was linked to mixed and diffuse type lesions. Nevertheless, the functional implications of these glycoproteins in the context of the different types of lesions is poorly understood and should be subject to more profound investigation in future studies. Moreover, all these glycoproteins were also tremendously overexpressed in healthy tissues limiting their potential for targeted therapeutics. Therefore, we have used an alternative strategy using an algorithm developed in-house exploiting information from the Human protein atlas. This algorithm, termed target score, was designed to pinpoint cancer biomarkers showing high cancer specificity and limited off-target potential due to low/null expression in healthy tissues. It balanced the expression of the proteins in healthy tissues *versus* cancer; its subcellular localization in tumours, privileging expression at the cell surface; and associations with poor prognosis. On the other hand, it severely penalized proteins showing wide distribution in healthy organs. Surprisingly the top-ranked protein was NCL, a protein commonly found in the nucleus of healthy cells and previously reported also at the cell membrane of esophageal [45], gastric [46], colorectal [47] and breast [48] cancer cells. Notably, most of the glycoproteins significantly overexpressed by gastric tumours were found in the lower part of the ranking, severely penalized by its healthy tissues' high levels, which suggested low potential for precise cancer targeting. We have then explored protein-protein networks provided by ClueGo and CluePedia for Cytoscape to gain more insights on their potential functional roles. In general, there is a significant level of interaction between the identified glycoproteins in the mediation of six main biological functions: i) differentiation; ii) cytoskeleton structure; iii) cadherin-mediated cell-cell adhesion; iv) protein targeting to the cell membrane; v) protein translation; and vi) cellular response to epidermal growth factor stimulus. Such observations support a conserved role for these glycoproteins in distinct cell models. Notably, NCL constituted an important hub protein in the regulation

of protein translation and, surprisingly, also in the mediation of response to epidermal growth factor, which is a key aspect in GC underlying progression and dissemination [49, 50]. Although the functional role of NCL in GC remains unknown, important observations were made to guide future studies on this field.

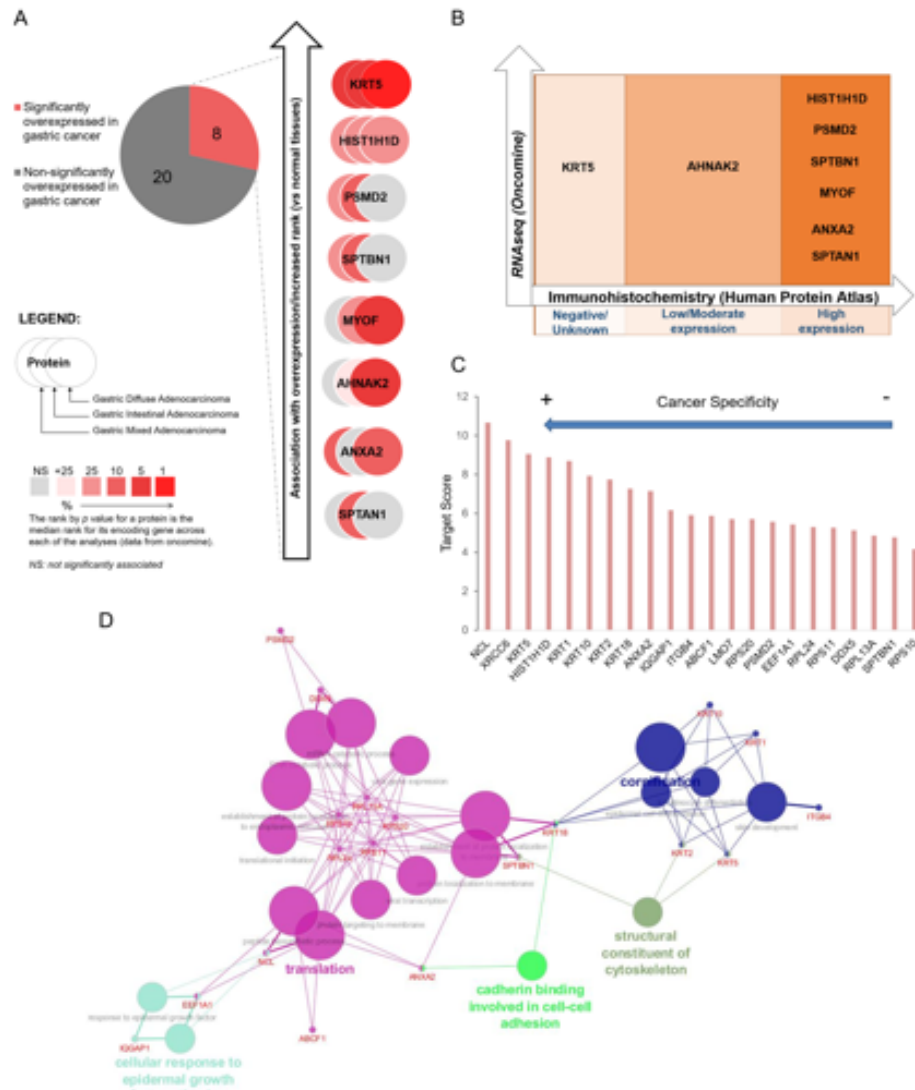


Figure 4. Bioinformatics analysis of the 22 glycoproteins expressing SLe^A and showing affinity to E-selectin that were present in all cell lines towards biomarker discovery.

A) Identification glycoproteins showing elevated gene expression. The 22 glycoproteins expressing SLe^A and showing affinity to E-selectin that were present in all cell lines were comprehensively matched against existing transcriptomics data in the OncoPrint database. Only 8 have been previously observed overexpressed in GC distributed according to intestinal, mixed and diffuse type lesions as highlighted in the graph in the right. Notably, *KRT5* and *HIST1H1D* were significantly overexpressed in all types, *PSMD2* and *SPTBN1* were mainly associated with the intestinal and mixed phenotype, whereas *MYOF*, *ANXA2* were

mainly overexpressed in diffuse lesions. **B) Comparison between transcripts and protein levels for the identified glycoproteins.** Transcriptomics findings (RNAseq; Oncomine) were matched against protein expression in GC (immunohistochemistry, Human Protein Atlas). KRT5 showed low/no expression at the protein level, thus not reflecting increased transcriptional activity. HIST1H1D, PSMD2, SPTBN1, MYOF, ANXA2, SPTAN1 showed both increased gene expression and high protein levels in GC. **C) Target score for the identified glycoproteins in GC.** Briefly, higher target scores are given to glycoproteins highly expressed at the cell membrane in cancer cells and showing low levels of expression in healthy tissues. Aspects related with poor prognosis significantly contribute to increase the scoring potential. Sub-cellular re-localization of proteins from intracellular compartments in healthy cells to the cancer cell membrane, an aspect that frequently occurs in cancer, is also highly score. In opposition, proteins showing a high expression in multiple healthy tissues are penalized in comparison to those showing a more cancer-specific expression pattern. According to this protocol, NCL (that was not found overexpressed in cancer tissues) ranked first as a potentially targetable biomarker due to its cancer specific nature. **D) Protein-protein networks highlighting the main biological functions played by the identified glycoproteins.** The map demonstrates a significant level of interaction between the identified glycoproteins towards different biological functions, namely cell differentiation, cytoskeleton organization, cell-cell adhesion mediated by cadherins and translation and mediation of responses to epidermal growth factor stimulus. Notably, NCL appears to play a key role in the latter two processes.

Nucleolin expression in gastric cancer cells

The possibility of SLe^A-glycoforms expression at the cell surface of GC cells was then validated by flow cytometry, immunofluorescence and immunoprecipitation assays followed by western blot, backed by protein identification by mass spectrometry. We have started by addressing the nature of NCL proteoforms by western blot in total cell lysates and cell membrane enriched extracts. According to **Figure 5**, all cell lines expressed NCL, thus in agreement with glycoproteomics studies. In addition, all presented similar western blot patterns, characterized by dominant signals at 100 kDa (the canonical form), 75 and 50 kDa. Several less intense bands were also evident below the 25 kDa mark, supporting the presence of shorter proteoforms at the cell surface. These observations were confirmed by NCL IP assays performed with the N87 cell line, which also revealed novel signals at approximately 150 and 250 kDa. Despite their low intensity, this

also highlighted the existence of macromolecular NCL species at the cell surface, most likely due to extensive glycosylation of the canonical form. Blotting for SLe^A demonstrated that this glycan was present in proteins found between 75 and 50 kDa as well as at 150 and 250 kDa, however not at 100 kDa. Such observations reinforced that SLe^A-NCL glycoforms presented a low molecular weight nature in comparison to its canonical form; even though the existence of heavily glycosylated proteoforms could not be excluded. Immunoprecipitation for SLe^A and blotting for NCL further reinforced these observations. Moreover, all identified signals were highly responsive to neuraminidase treatment, confirming the existence of sialylated Lewis antigens.

A comparative glycoproteomics study was then used to gain more insights on the type of NCL forms showing affinity for E-selectin. Briefly, the glycoproteins resulting from the SLe^A and E-selectin/IgG chimera IPs were analyzed in a molecular weight resolved manner. This confirmed the western blot assignments for NCL in the N87 cell line (main bands between 37-75 kDa), including the presence of SLe^A-glycopeptides. Notably, higher molecular weight glycoforms suggested by western blot could not be confirmed probably due to its low abundance. Moreover, E-selectin affinity was mediated by isoforms between 75-50 kDa. Similar analysis of the KATO-III cell line showed higher molecular weight glycoforms (75-150 kDa), with the glycoproteins showing affinity for E-selectin falling within the 75-100 kDa range. In clear contrast with N87, OCUM-1 and KATO-III cells expressed low molecular weight NCL-SLe^A glycoforms (<37 kDa), with glycoforms between 25-15 kDa showing affinity for E-selectin. Such observations suggest that different cell lines may express SLe^A-NCL glycoproteoforms of variable lengths and distinct affinities for E-selectin. The fact that NCL expression patterns is similar for all cell lines strongly suggests that marked differences in the glycosylation machinery of the cells translated by distinct glycomes may, in part, account for these findings.

Finally, we confirmed the co-localization of NCL and SLe^A at the cell membrane in all cell lines (**Figure 5**). This was further reinforced by positive *in situ* proximity ligation assays, denoting very close spatial proximity

between the glycan and the protein. Taken together with previous observations, these findings decisively demonstrated that GC cells expressed abnormally *O*-glycosylation NCL at the cell membrane presenting the SLe^A antigen.

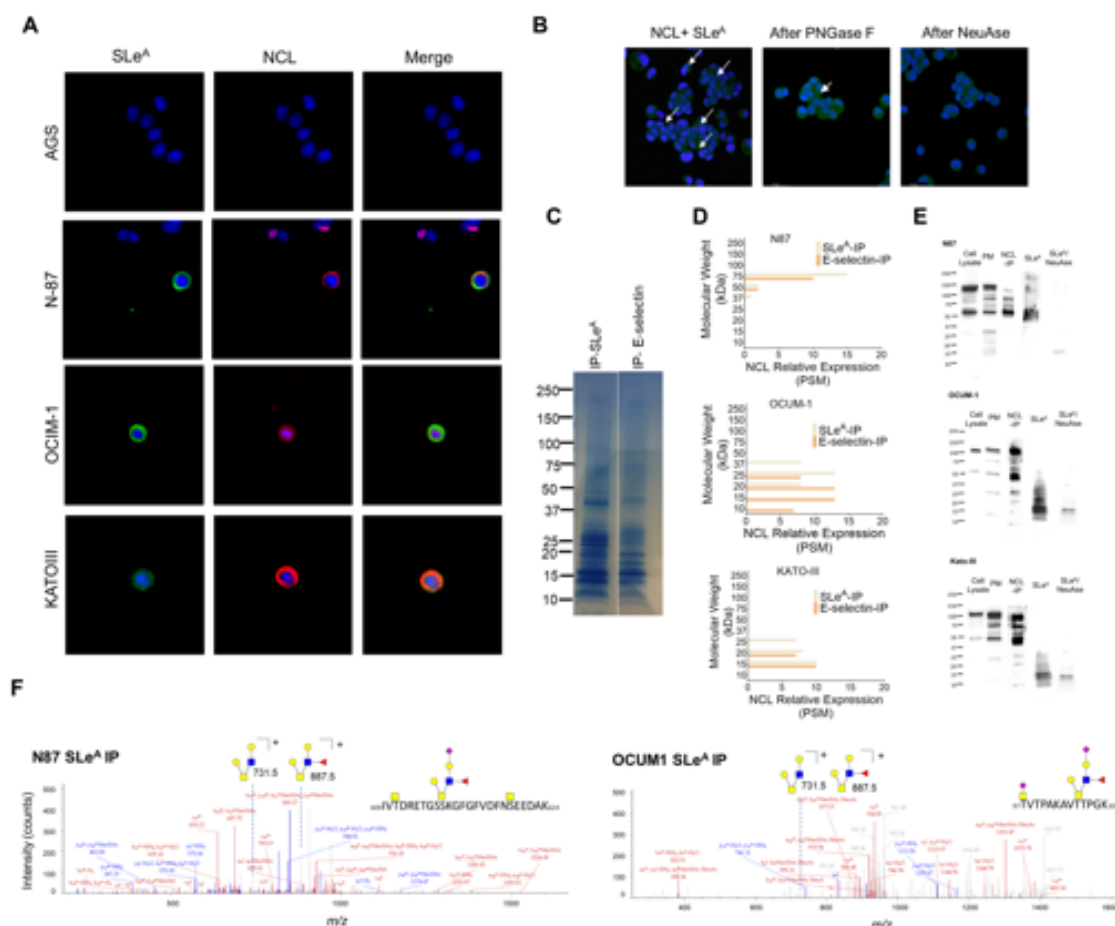


Figure 5. Identification of SLe^A-NCL glycoforms in gastric cancer cell models. A) Immunofluorescence staining of GC cells (AGS, N87, OCUM-1, KATOIII) for SLe^A and NCL. Neither SLe^A nor NCL were detected in AGS cell lines. However, clear cell membrane staining was observed for both antigens in N87, OCUM-1 and KATOIII. B) PLA assays for SLe^A-NCL in N87 cells. Positive PLA signals (white arrows) were observed for N-87 cell lines, that significantly decreased with PNGase F digestion and completely disappeared in cells digested with neuraminidase. C) Typical SDS-PAGE for glycoproteins isolated by IP for SLe^A and E-selectin. The gels were an important starting point for molecular weight resolved identification of NCL in GC cells. Briefly, bands were excised from the gels according to the exhibited molecular weights, reduced, alkylated, digested with trypsin and analysis by nanoLC-ESI-MS/MS. D) NCL expression according to molecular weight resolved glycoproteomics analysis. The graphs show that for N87 cell lines NCL is mainly present in 75-50 kDa forms, whereas for OCUM-1 and KATOIII NCL is present at much lower

molecular weights (37-10 kDa). Notably, IP-SLe^A and IP-E-selectin showed similar NCL patterns. **E) Western blots for NCL in total cell lysates (cell lysate), plasma membrane (PM) extracts and NCL IPs and for SLe^A in NCL IPs before (SLe^A) and after neuraminidase digestion (SLe^A/NeuAse; used as negative control).** A similar NCL expression pattern could be observed for all cell lines in cell lysates and PM extracts characterized by dominant band just above 100 kDa, 75 and 50 kDa and low intense signals of lower molecular weights. However, IP for NCL suggests different patterns according to the cell line. N87 presented a more intense band at approximately 50 and 75 kDa, whereas OCUM-1 and KATOIII presented similar patterns characterized also by high expression of the band at 100. Moreover, the IPs for both cell lines reinforced the presence also of lower molecular weight NCL species (>50 kDa). Notably, in N87 cell lines SLe^A-NCL glycoforms appear as a smear between 75-50 kDa while for OCUM-1 and KATOIII the smear was observed bellow 50 kDa, with emphasis between 25-15 kDa. All these signals were highly responsive to neuraminidase and support molecular weight resolved proteomics findings. Taken together, these observations support the existence of similar NCL proteoforms in all cell lines, with distinct SLe^A glycopatterns according to the cell line. **F) MS/MS for SLe^A-NCL glycopeptides identified by MW-resolved glycoproteomics in N87 and OCUM-1 cell lines.** Tandem spectra support the existence of glycopeptide fragments carrying the SLe^A antigen in the bands corresponding to NCL-IPs between 75 and 50 kDa for the N87 cell line and between 25-37 for OCUM-1.

Nucleolin expression in gastric cancer and healthy tissues

We have then screened for SLe^A and NCL a small cohort of gastric tumour sections comprehending distinct clinicopathological types (9 intestinal, 1 mixed, 5 diffuse) as well as 7 lymph node metastases. The main objective was to disclose the strong cancer associated nature suggested by glycomics, glycoproteomics and comprehensive bioinformatics studies. The SLe^A antigen was widely expressed in most primary tumours (80%; **Figure 6A**) and in approximately half of the metastasis, reinforcing the glycan's know functional role in this process. The SLe^A antigen exhibited intense membrane expression, being present in more than 25% of the tumour area, irrespectively of its clinicopathological classification. On the other hand, NCL was extensively expressed in the nucleus of most cells in all cases (**Figure 6A**). However, half the tumours also presented scattered cancer cells (not exceeding 5% of the lesion) with marked membrane expression accompanied by the loss of nuclear expression (**Figure 6A**). In primary

tumours, a trend association was observed between the presence of NCL at the cell membrane and the intestinal subtype (67% of positive cases in the intestinal subtype; 1 negative mixed subtype; 20% of positive cases in the diffuse subtype). Membrane NCL was further observed in 57% (4/7) of lymph node metastasis, suggesting a possible role in this process.

In summary, these observations support that NCL is expressed at the cell membrane, associated to a subpopulation of cells of yet unknown role in the disease. Future studies must devote to understanding the clinical significance of NCL expression at the cell membrane, including its potential association with the intestinal subtype, prognosis, response to distinct therapeutic schemes and contribution to clinical management, namely by incorporation in currently accepted molecular disease classifications. Emphasis should also be set on disclosing the functional impact of cell surface NCL. We have also found that 68% of primary tumours and metastasis expressing NCL concomitantly expressed SLe^A antigen, with relevant co-expression in the same tumour areas, supporting the co-localization observed in cancer cell lines.

Foreseeing precise cancer targeting, we have also evaluated both SLe^A and NCL in a wide array of relevant healthy tissues. SLe^A was observed in the cytoplasm of exocrine pancreas cells, suggesting association to secreted proteins (**Figure 6B**). It was also mildly detected at the cell membrane of goblet cells of the gastrointestinal tract, cells composing the upper layers of the corneal epithelium, rare thyroid parafollicular cells and pulmonary secretions (**Figure 6B**). In turn, nuclear NCL was abundantly observed in all tissues. On the other hand, membrane protein expression was observed in corneal epithelium, not in the same cell types expressing SLe^A, and epidermis (**Figure 6**). These observations suggest that the conjugation of SLe^A and NCL at the cell membrane is a rare event associated with cancer, holding cancer-specific targeting potential. Nevertheless, screening a wider array of healthy tissues is still required to fully characterize SLe^A-NCL glycoforms expression patterns in health and disease.

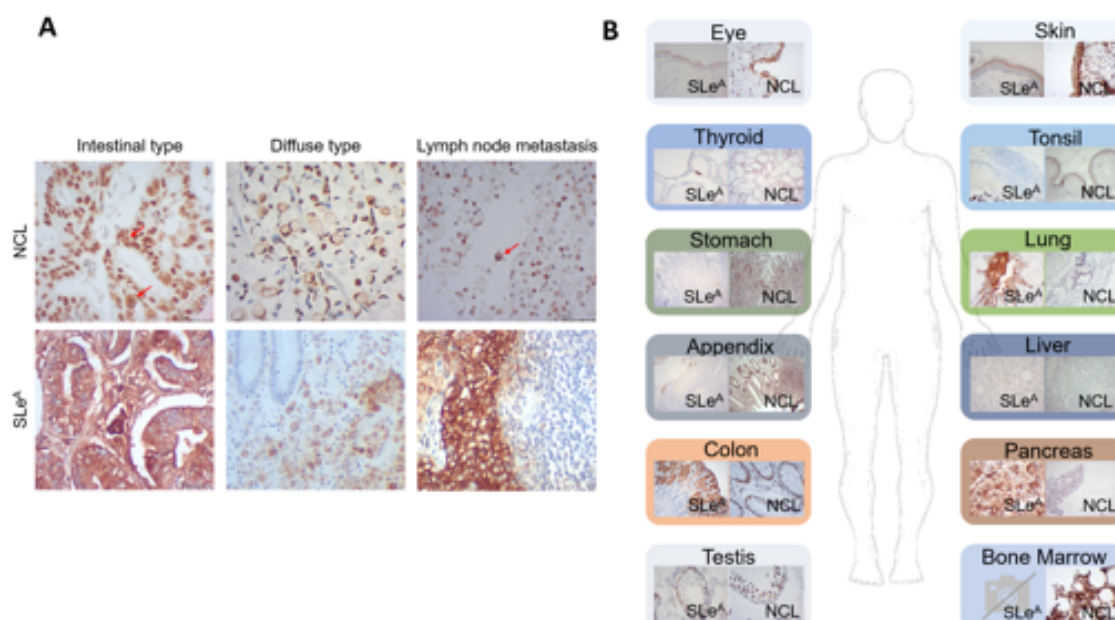


Figure 6. A) SLe^A and NCL expressions in intestinal, mixed and diffuse type GC and lymph node metastasis. Both SLe^A and NCL could be at the cell membrane of gastric cancer cells in intestinal, mixed and diffuse type tumours, in co-localization, reinforcing the notion of SLe^A-NCL glycoforms. Notably, NCL could be observed in both the nucleus (its normal location in healthy cells) as well as the membrane of cancer cells. This could also be observed in the metastasis. **B) Expression of SLe^A and NCL in healthy human tissues of relevant organs.** SLe^A was found in the cytoplasm of exocrine pancreas cells and mildly detected at the cell membrane of goblet cells of the gastrointestinal tract, cells composing the upper layers of the corneal epithelium, rare thyroid parafollicular cells and pulmonary secretions. NCL was detected in the nucleus of most healthy cells. Membrane NCL was observed only in corneal epithelium, not in the same cell types expressing SLe^A, and the epidermis. These observations suggest that the conjugation of SLe^A and NCL at the cell membrane is a rare event associated with cancer, holding cancer-specific targeting potential.

Concluding remarks

The tremendous advances in the molecular subtyping of gastric tumours over the past five years have significantly contributed to improve its clinical management, enabling better therapeutic selection and prognosis [9, 10, 51]. However, the quest for molecular signatures associated to metastatic spread persists as a daunting research topic due to significant intra- and inter-tumour molecular heterogeneity. Exploring alterations in

protein glycosylation occurring at the cancer cell surface holds tremendous potential towards this end [13, 14].

Based on these observations we have focused on glycoproteins expressing the SLe^A antigen, a well-known mediator of metastasis that allows GC cells to bind E-selectin on activate endothelial cells, supporting their extravasation into the blood and lymph and, ultimately, intravasation into several organs [52-55]. Accordingly, we have identified the SLe^A in both *N*- and *O*-glycoproteins and specific *O*-glycome signatures associated with this modification. Nevertheless, despite its key functional role, SLe^A was residually expressed in comparison to other types of *O*-glycans, denoting a highly restricted phenotype possibly associated to more aggressive cell subpopulations, as suggested by different studies [23, 56]. This structural information also enabled the identification of a wide number of glycoproteins, significantly broadening our understanding on the SLe^A-glycoproteome. Glycoproteomics studies clearly showed that the SLe^A-glycoproteome varied significantly with the type of cell and have highlighted marked cell-type dependent glycoproteomes, reinforcing the need for personalized molecular characterization at this level. However, only a small percentage of the identified glycoproteins showed affinity for E-selectin, suggesting that the presence of SLe^A is not a sole determinant for this process. Future studies must now comprehensively disclose how the density and distribution of glycosites, the structure and type of glycans chains as well the nature of the protein may influence E-selectin recognition. These aspects will be critical for elucidating the molecular basis mediating the interaction between GC cells with endothelial cells. Notably, sialylated Lewis glycan mimetics are currently being used in pre-clinical and clinical settings to inhibit E-selectin mediated metastasis [20, 57]. A more profound knowledge about the molecular aspects governing this interaction will be a key milestone towards improving this emerging therapeutic strategy.

Finally, we observed that only a small subset of 22 SLe^A-expressing glycoproteins with affinity for E-selectin were present in all cell lines. Our in-house target score algorithm was then used to rank these glycoproteins according to their cancer-specificity. Surprisingly, NCL, a typical nuclear

protein found generally associated with intracellular chromatin and playing a central role in polymerase I transcription in healthy cells [58], emerged as a top-ranked cancer protein at the cell membrane. Its cell membrane location in cancer cells and not in the nucleus, as generally occurs in healthy cells, significantly contributed to this stratification. Interestingly, the presence of NCL at the cell-surface was described for the first time over thirty years ago [59] and has been since then observed in cancer cells of different origins [58] including in GC [60, 61]. Nevertheless, these observations remain quite surprising since the protein does not present transmembrane hydrophobic domains nor a plasma membrane targeting sequence [58]. Moreover, it has been found glycosylated by an yet unraveled molecular mechanisms [62, 63], with glycosylation playing a fundamental role in its biological function [62-64]. Namely, at the plasma membrane, NCL interacts with several membrane and external ligands involved in cell proliferation, differentiation, adhesion, mitogenesis and angiogenesis [58]. In GC, membrane NCL has also been found to mediate the incorporation of tumour necrosis factor- α (TNF- α)-inducing protein, produced in large amounts by *Helicobacter pylori*. The NCL-TNF α -inducing protein complex was shown to promote epithelial-mesenchymal transition of cancer cells, inducing cell migration and invasive morphological changes [65]. On the other hand, in the nucleus, NCL plays a relevant role maintaining genome stability [66]. Therefore, in cancer cells, nuclear NCL overexpression is considered critical to control the balance between DNA replication and repair, limiting DNA damage accumulation due to high proliferation rates [58]. Supporting these observations, high levels of nucleolar NCL was demonstrated to be an independent prognostic marker for better survival in GC, while high cytoplasmic staining was closely associated with worse prognosis [67]. Nevertheless, the clinical significance of its translocation to the cell membrane has not yet been disclosed in the context of GC. However, it is possible that NCL abnormal subcellular localization at the cell membrane may promote a more aggressive phenotype, as suggested by its functional role in GC. Notably, our bioinformatics analysis associated NCL, together with other identified glycoproteins, to cellular responses to

epidermal growth factor stimulus. Supporting these observations, NCL has been found to stabilize ErbB1 and interact with Ras. The three proteins act synergistically to mediate tumor growth and favour cancer cell proliferation and survival [68, 69]. Our observations suggest that NCL may also be involved in ErbB1-mediated signaling in GC, even though the exact mechanism remains unknown, warranting further investigation.

Another striking observation is that NCL at the cell membrane is targeted by E-selectin, strongly suggesting its glycoforms may play a key role in disease dissemination, which should be addressed in future studies. Surprisingly, these glycoforms were of low molecular weight compared to the canonical form. It is likely that altered splicing mechanisms accompanying disease progression may lead to the expression of truncated forms, which warrants also careful evaluation in future studies envisaging to understand their functional role in cancer. Finally, we have screened a wide number of relevant healthy tissues for membrane NCL and SLe^A antigen. According to our observations, sole targeting of membrane NCL or SLe^A could potentiate off-target effects negatively affecting ocular, cardiac and gastrointestinal organs. Contrastingly, the bi-specific nature of SLe^A-NCL glycoforms may pave the way for precise cancer targeting, including means to address both primary lesions and the metastasis.

In summary, this work sets a novel molecular rationale for targeting gastric cells of higher metastatic potential translated by increased affinity for E-selectin. It also suggests that the overexpression of SLe^A-NCL glycoforms at the cell surface of cancer cells may be a key mediator of this process. The cancer-specific nature of this alterations also supports potential for precise cancer targeting with limited off-target effects in healthy organs. It is now becoming crucial to elucidate the functional impact of such observations as well as their clinical context foreseeing improved strategies for controlling disease dissemination.

Conflict of interests

The authors declare no conflict of interests

Acknowledgments

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CONCLUDING REMARKS & FUTURE PERSPECTIVES

Concluding Remarks & Future Perspectives

GC is the third leading cause of cancer-related death worldwide, with an estimated 783,000 deaths in 2018 [1]. The efficacy of mainstay therapeutics is constantly challenged by significant intra-tumoral clonal heterogeneity associated to multiple intrinsic and acquired resistance mechanisms and dose-limiting side-effects [2]. Thus, targeted therapeutics are warranted to improve treatment outcomes and reduce toxicity. Malignant transformation is accompanied by significant alterations in cell surface glycosylation holding tremendous potential for cancer detection and targeted therapeutics. Advanced gastric tumours are characterized by the overexpression of SLe^A and STn glycobionarkers as well as loss of epithelial adhesion promoted by E-cadherin molecules and Ki-67 proliferation marker overexpression [3-5]. Accordingly, we reinvestigated these clinically relevant markers in a representative gastric tumour tissue series envisaging patient's stratification according to pertinent clinicopathological variables. Specifically, aberrantly glycosylated, poor proliferative and less cohesive tumours have emerged as particularly aggressive, which translated in significantly decreased patient's CSS. Moreover, such tumour phenotypes defined poor prognosis subgroups within favourably diagnosed patients, such as M0 and N0 according with the TNM staging system. These findings provide a roadmap to identify patients which could benefit from closer follow-up and more aggressive therapy. Moreover, the cell surface nature of these glycobiomarkers also provides means to address targeted therapy to these patients. Finally, these findings highlight the need for the refinement of broadly used GC classifications with molecular data capable of guiding clinical decision, while allowing monitoring of therapeutic response throughout the course of patient's management.

Given the cell surface and cancer-specific nature of SLe^A, it constitutes an ideal target for drug delivery. As such, the present work devoted to the development of a novel biocompatible PLGA-based nanoparticle targeting aggressive SLe^A positive GC cells to safely deliver chemotherapy. Nanoparticle functionalization significantly enhanced targeting to SLe^A

positive cells *in vitro* and *ex vivo*, while reducing the upfront cytotoxicity of chemotherapeutic agents.

Exploiting the sialyl-lewis A (SLe^A) antigen and glycoproteins with affinity for E-selectin in different gastric cancer cell models, foreseeing the identification of molecular features associated with metastasis development by mediating cancer cells adhesion to epithelial cell. An in-house bioinformatics-assisted algorithm identified nucleolin (NCL), a nuclear protein frequently in healthy cells, at the membrane of gastric cancer cells and expressing SLe^A, as a potential targetable biomarker. We have demonstrated that the miss-localization of nuclear NCL to the cell surface may be used to selectively target cancer cells presenting higher metastatic potential translated by higher affinity to E-selectin. Moreover, the generated glycoproteomics information may now be used as starting point to improve the specificity of SLe^A-based targeted therapeutics.

Overall, this work provides a proof-of-concept therapeutic approach vowing for glycan-based therapeutic solutions to target aggressive GC phenotypes. Furthermore, since abnormal SLe^A and STn expressions seem to be intricately correlated, combined targeted therapeutic strategies could improve clinical response compared to monotherapies, which warrants careful evaluation. In addition, STn expression correlated with lymph-node metastasis, while SLe^A expression was associated with hematogenous metastasis, suggesting distinct metastatic pathways for these two glycobiomarkers. Accordingly, the mechanistic involvement of these carbohydrate chain in preferential metastatic routs should also be thoroughly addressed. Afterwards, we believe that the necessary rationale has been created to foster more in-depth studies on the glycobiology of GC envisaging molecular-based precision medicine and improved patient care.

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