ImmunoTools *FlowISiAM* Award 2026

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Flow Cytometric Immune Profiling of HPV-Driven Oesophageal Adenocarcinoma (AMANDUS-OAC): Identification of Prognostic Biomarkers Using FlowISiAM

Background and Rationale

Oesophageal adenocarcinoma (OAC) is an aggressive malignancy with a rapidly increasing incidence and a five-year survival rate of approximately 15%, highlighting an urgent need for improved biological stratification and prognostic tools [1, 2]. Over the past decade, our research program has fundamentally redefined OAC pathogenesis by demonstrating that a substantial subset of OAC is driven by transcriptionally active high-risk human papillomavirus (HPV), and that HPV-positive disease is consistently associated with superior long-term survival [3, 4].

Subsequent genomic analyses revealed that HPV-positive OAC represents a biologically distinct entity characterised by wild-type TP53 and a reduced somatic mutational burden [5, 6], suggesting fundamentally different tumour–host immune interactions. Building on this foundation, our recent tissue-based immune profiling study demonstrated that HPV-negative OAC exhibits a profoundly immunoregulatory tumour microenvironment, marked by increased FoxP3^{+} regulatory T-cell (Treg) infiltration and significantly reduced CD8^{+}:Treg ratios, which independently predict poor overall survival [7].

Existing HPV biomarker studies predominantly focus on viral DNA detection, serological responses, and lymphocyte phenotyping [8, 9]; however, recent mechanistic studies demonstrate that the HPV E6 and E7 oncoproteins actively reprogram intracellular transcriptional, metabolic, and inflammatory pathways within macrophages and monocytes, including pathways governing cytokine regulation, interferon signalling, and cellular metabolism [10-12]. These intracellular immune pathways remain unexplored in circulating immune cells, establishing a strong rationale for *FlowISiAM*-**based intracellular monocyte profiling**.

Experimental Design

This study will apply the *FlowISiAM* **platform** to perform single-cell intracellular profiling of circulating monocytes in patients with HPV-driven OAC, integrating viral, immune, and functional tumour-associated readouts from peripheral blood. Whole blood will be collected in EDTA tubes and processed within 4 hours using the validated *FlowISiAM* whole-blood workflow, avoiding prior cell isolation to preserve physiological immune cell states.

Circulating monocytes will be identified based on forward and side scatter characteristics in combination with CD14 and CD16 surface expression, enabling discrimination of classical and non-classical monocyte subsets. Following surface staining, cells will be fixed and permeabilised using a *FlowISiAM*-validated protocol optimised for intracellular antigen detection.

A **six-marker intracellular panel** will be employed capturing transcriptional (KLF2), metabolic (HIF-1α, phospho-mTOR, TKTL1), inflammatory (NF-κB p65), and apoptosis-related (Apo10) reprogramming of circulating monocytes in HPV-driven disease (*FlowISiAM-OAC*). Apo10 and TKTL1 are incorporated as validated pan-cancer markers of dysregulated apoptosis resistance and altered glucose metabolism, respectively, and are detected intracellularly within phagocytosing immune cells using the *FlowISiAM* AT-test. Together, these markers integrate **HPV-specific immune modulation with general tumour-associated functional signatures**, representing a largely unexplored biomarker layer in circulating immune cells.

Data acquisition will be performed using standardised *FlowISiAM* acquisition settings on a multi-parameter flow cytometer. Analysis will include sequential gating of singlets, viable leukocytes, and CD14/CD16-defined monocytes, with intracellular marker expression quantified as median fluorescence intensity and proportion of biomarker-positive cells. Fluorescence-minus-one controls will be included to ensure robust signal discrimination.

This experimental design enables the detection of phagocytosed and intracellularly processed protein signatures within circulating monocytes, providing a functional immune and tumour-associated readout that complements HPV DNA, E6 antibody, and lymphocyte-based biomarkers. By introducing intracellular monocyte profiling into HPV biomarker research, this study addresses a critical knowledge gap and establishes a clinically scalable platform for integrated blood-based immune monitoring.

Data Analysis and Clinical Correlation

Immune cell frequencies, phenotypes, and ratios will be compared between HPV-positive and HPV-negative OAC cohorts. Particular emphasis will be placed on:

- $CD8^+$:Treg ratios, previously shown to be independently prognostic
- Differences in circulating Treg abundance
- Presence of TRM-like circulating T-cell populations
- Associations with overall survival, disease progression, and treatment response

Statistical modelling will assess whether circulating immune signatures recapitulate tumour microenvironment findings and whether they can function as surrogate biomarkers of tumour immune status.

Aim

To define circulating immune cell signatures that mirror tumour immune phenotypes identified in HPV-driven OAC and to determine their prognostic value using multiparametric flow cytometry.

Innovation

This project is innovative because it:

- Directly translates recent high-impact tissue-based discoveries (Wu et al., *Journal of Translational Medicine*, 2026) into a minimally invasive and scalable platform
- Applies flow cytometry, a clinically established and widely accessible technology, to capture HPV-associated immune phenotypes
- Focuses on immune balance metrics ($CD8^+$:Treg ratios) previously shown by our group to be independently prognostic
- Explores circulating TRM-like and exhausted T-cell populations as surrogates of tumour immune status

Expected Outcomes

Expected outcomes include:

- Identification of circulating immune biomarkers reflecting the immunoregulatory tumour microenvironment described in our recent publication
- Validation of $CD8^+$:Treg imbalance as a systemic marker of poor prognosis in HPV-negative OAC
- Development of a flow cytometry biomarker panel suitable for longitudinal monitoring and risk stratification

Collectively, these outcomes will extend our published findings from observational tissue analysis to clinically actionable immune diagnostics.

Significance

By building directly on our demonstration that immune balance—rather than individual immune subsets—determines prognosis in OAC, this project advances the field beyond descriptive immunology toward translational implementation.

Successful completion will enable:

- Non-invasive immune risk stratification
- Improved patient selection for immunotherapy
- Rational treatment de-escalation for HPV-positive disease

Importantly, this work establishes a translational pipeline applicable across other HPV-driven malignancies.

Clear Statement of Continuation

This application represents the next translational phase of our recently published work, shifting from tumour tissue–based discovery to clinically scalable, flow cytometry–based immune profiling.

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ImmunoTools *FlowISiAM* AWARD for

Mohammad Rabiei and **Shanmugarajah Rajendra** includes

our antibodies for *FlowISiAM*, know how transfer and protocol, support regarding selection of specific antibodies against some specific biomarkers from INVIGATE, engagement regarding development of specific *AMANDUS-OAC* as well as *FlowISiAM-OAC* and expert assistance in evaluating the results obtained, and integration into the ImmunoTools *FlowISiAM* network.