

Squamous cell carcinoma of the anus: morphological evaluation and ancillary techniques

Carcinoma squamocellulare dell'ano: valutazione morfologica e tecniche ancillari

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Abstract

Background: the present study deals with anal carcinoma, focusing on morphological evaluation, histopathological characteristics, and immunophenotypic profile, as well as the incidence, risk factors, and molecular profile of this pathology, underlining the importance of a correct differential diagnosis.

Materials and Methods: this study was conducted in the laboratory of Anatomic Pathology at the Santa Rita Clinic, Monza Policlinic Group, located in Vercelli. We collected samples of 13 anal biopsies with a diagnosis of squamous cell carcinoma of the anus, referred from April 2022 to February 2025. A retrospective analysis was performed on paraffin-embedded Hematoxylin and Eosin (H&E) and immunohistochemical samples. The study contributed to expanding the case history present in the literature and allowed for a review of the differential diagnostic criteria in light of the immunophenotypic expression of neoplastic subtypes. The morphological analysis was performed by microscopic observation of the hematoxylin and eosin-stained specimen slide, while immunophenotypic analysis was performed by immunohistochemical staining of the specimen with specific antibodies (anti-pan keratin, anti-keratin, anti-CK8/18, anti-CK7, anti-p16INK4a, anti-p40, GATA3, anti-chromogranin A, anti-synaptophysin) and subsequent microscopic examination. Morphological analysis allowed for the identification of histotypes, while immunohistochemical analysis allowed for a differential diagnosis with other anal canal tumors.

Results: from the analysis of the case history, it was seen, in agreement with other previous studies, that this pathology is rare, as it is found in 3% of biopsies of the anal region, that it has a higher incidence in females, and, in the majority of cases, the neoplasm is related to Human Papillomavirus (HPV) infection.

Conclusions: anal squamous cell carcinoma is a rare but increasing neoplasm, with variable histological characteristics that can lead to misdiagnosis. Therefore, a correct differential diagnosis through analysis of morphology and immunophenotypic expression is essential. HPV infection is a significant risk factor, and vaccination in at-risk populations could reduce its incidence.

Background: il presente studio si focalizza sulla valutazione morfologica del carcinoma anale, analizzando le caratteristiche istopatologiche ed il profilo immunofenotipico oltre che l'incidenza, i fattori di rischio ed il profilo molecolare di tale patologia, sottolineando l'importanza di una diagnosi differenziale corretta.

Materiali e Metodi: lo studio è stato condotto presso il laboratorio di Anatomia Patologica della Clinica S. Rita, Gruppo Policlinico di Monza presso Vercelli. Abbiamo raccolto i campioni di 13 biopsie della regione anale presentanti una diagnosi di carcinoma squamocellulare dell'ano refertati nel periodo compreso tra Aprile 2022 e Febbraio 2025. È stata attuata un'indagine retrospettiva sui campioni istologici colorati in ematossilina ed eosina e sui campioni in cui è stata attuata una colorazione immunoistochimica. Lo studio ha contribuito ad ampliare la casistica presente in letteratura e ha permesso di rivedere i criteri della diagnostica differenziale alla luce dell'espressione immunofenotipica dei sottotipi neoplastici. L'analisi morfologica è stata effettuata tramite osservazione al microscopio del vetrino del campione colorato in ematossilina ed eosina mentre l'analisi immunofenotipica è stata effettuata mediante colorazione immunoistochimica del campione con specifici anticorpi (anti-pan keratin, anti-keratin, anti-CK8/18, anti-CK7, anti-p16INK4a, anti-p40, GATA3, anti-chromogranin A, anti-synaptophysin) e la successiva visione al microscopio. L'analisi morfologica ha permesso di individuare gli istotipi mentre l'analisi immunoistochimica ha permesso di attuare una diagnosi differenziale con altri tumori del canale anale.

Risultati: dall'analisi della casistica si è visto, in accordo con altri studi precedenti, che tale patologia risulta rara in quanto riscontrato nel 3% delle biopsie della regione anale, che abbia un'incidenza maggiore negli individui di sesso femminile e che nella maggior parte dei casi la neoplasia risulta correlata ad infezione da papillomavirus.

Conclusioni: il carcinoma squamocellulare anale è una neoplasia rara ma in aumento, con caratteristiche istologiche variabili che possono portare a una diagnosi errata e per tale motivo risulta fondamentale una corretta diagnosi differenziale mediante l'analisi della morfologia e dell'espressione immunofenotipica. L'infezione da papillomavirus è un importante fattore di rischio, e la vaccinazione nelle popolazioni a rischio potrebbe ridurne l'incidenza.

Key words: anal cancer, SCCA, Immunohistochemistry, HPV, p16.

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Introduction

Squamous Cell Carcinoma of the Anus (SCCA) is a malignant lesion of the epithelial lining. This condition has a broad clinical presentation, and about 25% of patients are asymptomatic.^{1,2} According to GLOBOCAN statistics, this neoplasm affects women more frequently than men, and the median age at diagnosis is 60 years.³ The etiopathological agent of anal squamous cell carcinoma has been identified as infection with Human Papillomavirus (HPV).⁴

HPV integrates into the genome and leads to the production of the viral oncoproteins E6 and E7, which cause the degradation and functional loss of p53 and pRb. This not only promotes uncontrolled cell proliferation but also induces instability, resulting in the accumulation of mutations and thus the possible development of neoplasms, including SCCA.⁵⁻⁷

However, the risk of developing the neoplasm is associated with the presence of risk factors such as sexual behaviors, immunodeficiency in individuals with Human Immunodeficiency Virus (HIV), immunosuppression following organ transplantation, and smoking.¹

In a meta-analysis, several risk groups and the incidence rates as cases per 100 000 person-years of anal cancer within have been identified. The results of this study are reported in Table 1.⁸

SCCA often presents with bleeding, a symptom that is frequently attributed to the presence of hemorrhoids, leading to possible delays in diagnosis.⁹ Early diagnosis of anal cancer can improve outcomes; therefore, when there is clinical suspicion, it is important to follow a proper diagnostic algorithm. This includes a physical

examination, digital anorectal exploration, and biopsy for histopathological analysis. In the case of a positive result, imaging techniques such as computed tomography, magnetic resonance imaging, and endoanal ultrasound should be performed to enable more detailed staging. It is also essential to test for possible HIV or HPV infection.¹⁰

Biopsy of any suspicious area within the anal canal or at the anal margin is recommended, and enlarged inguinal lymph nodes must be pathologically assessed.¹¹

Hematoxylin and eosin staining highlights the morphological features. Patterns may include eosinophilic cells with keratinization, small cells with palisading nuclei, tubular structures, or spindle-cell components. None of these features have shown prognostic significance; therefore, immunohistochemical staining is required.¹

The immunoexpression panel recommended by the WHO for the diagnosis of anal squamous cell carcinoma is presented in Table 2.¹

Another essential immunohistochemical staining is that for p16, as the expression of the p16 protein usually indicates infection with oncogenic HPV. The p16 gene encodes a cell cycle inhibitor protein, and its overexpression is frequently observed in HPV-associated tumours, such as squamous cell carcinoma of the anus. P16 staining is used in differential diagnosis to distinguish HPV-associated squamous cell carcinomas from other anorectal neoplasms.¹

Immunohistochemical staining is important for differential diagnosis in order to rule out other tumours in the anal canal. Neoplasms that are differential diagnoses for squamous cell carcinoma of the anus are summarized in Table 3.¹

Table 1. Incidence rates of Squamous Cell Carcinoma of the Anus in risk groups.

Risk groups		Incidence rates
HIV-positive MSM		85
HIV-positive non-MSM		32
HIV-positive female		22
HIV-negative MSM		19
Women diagnosed with HPV-related gynecological precancerous lesions	Vulvar	48
	Cervical	9
	Vaginal	10
Solid organ transplant recipients		13
Patients with autoimmune diseases	Lupus erythematosus	10
	Ulcerative colitis	6
	Chron's disease	3

HIV, Human Immunodeficiency Virus; MSM, Men who have Sex with Men, HPV, Human Papilloma Virus.

Table 2. Immunohistochemistry pattern for Squamous Cell Carcinoma of the Anus.

8+18	Keratins			Mucin	CEA	Vimentin
	7/20	5+14	13/19			
-	-/-	+	+/+	-	-	-

CEA, carcino-embryonic antigen.

Table 3. Immunohistochemistry pattern for anal tumours.

Tumour	Keratins			Mucin	CEA	Vimentin	Others
	8+18	7/20	5+14				
Adenocarcinoma	+	-/+	-	+	+	-	/
Basal cell carcinoma	-	-/-	+	-	-	-	Ber-EP4
Neuroendocrine tumour	+	-/-	-	-	-	-	Chrom, Synap
Melanoma	-	-/-	-	-	-	+	S100, HMB45
Bowen	-	-/-	+	-	-	-	/
Paget cells (local)	+	+/-	-	+	+	-	GCDPF-15
Paget cells (secondary)	+	+/+	-	+	+	-	/
Prostatic carcinoma	+	-/-	-	-	-	-	PSA, PSAP
Lymphoma	-	-/-	-	-	-	+	LCA

CEA, carcino-embryonic antigen.

Treatment of this neoplasm initially involved a surgical approach; however, chemotherapy associated with radiotherapy is now preferred. There is no specific immunotherapy for this neoplasm, but it may be used in some patients with associated PDL1 or MSH protein mutations.¹²⁻¹⁴

Materials and Methods

The cases were reviewed using the data archive platform of the Pathology Department, selecting, through an epidemiological investigation of the period between April 2022 and February 2025, the morphological inclusion criterion “Anus, NAS.” From the results obtained, only data related to samples from patients diagnosed with squamous cell carcinoma were selected. A retrospective analysis of these cases was then performed, examining the respective hematoxylin and eosin-stained slides and those tested with immunohistochemical analysis.

To describe the morphology of the selected cases, the Hematoxylin and Eosin (H&E)-stained slides were observed under a light microscope at various magnifications (from 5x to 40x). Based on the microscopic evaluation and morphological description, squamous cell carcinoma was classified into three histotypes: keratinizing large-cell subtype, non-keratinizing large-cell subtype, and basaloid subtype.

Subsequently, immunophenotypic analysis of the selected samples was carried out. This analysis was performed through immunohistochemical staining using specific antibodies on the Ventana BenchMark ULTRA immunostainer, followed by microscopic examination.

The previously processed and mounted biopsy samples were loaded onto the immunostainer and underwent staining through multiple steps: deparaffinization, incubation with primary antibodies, addition of secondary antibodies or detection systems, staining, and counterstaining. This allowed visualization of cellular components such as the nucleus, cytoplasm, or cell membrane, depending on the localization of the antigen of interest. The detection technology used specific chromogens for the target antigens; in this study, the detection system used was the OptiView DAB IHC Detection Kit, which generates a brown signal.

The antibodies used for the differential diagnosis of anal neoplasms included: Ventana anti-pan keratin (AE1/AE3/PCK26) pri-

mary antibody, Ventana CONFIRM anti-keratin (34βE12) mouse monoclonal primary antibody, Ventana anti-CK8/18 (B22.1 and B23.1) mouse monoclonal primary antibody, Ventana anti-CK7 (SP52) rabbit monoclonal primary antibody, Ventana CINtec® anti-p16INK4a (E6H4) mouse monoclonal primary antibody, Ventana GATA3 (L50-823) mouse monoclonal primary antibody, Ventana anti-chromogranin A (LK2H10) primary antibody; Ventana CONFIRM anti-synaptophysin (SP11) rabbit monoclonal primary antibody.

To confirm the epithelial origin of the neoplasm, immunohistochemical staining with four antibodies targeting cytokeratins was performed. The Ventana anti-pan keratin antibody, a combination of three monoclonal antibodies, recognizes Cytokeratins (CK), whose expression indicates an epithelial lesion since CKs are surface markers of epithelial cells. Therefore, CK positivity is observed in all carcinomas, including anal squamous cell carcinoma. According to the World Health Organization (WHO) immunoprofile for SCCA, this neoplasm typically lacks low-molecular-weight cytokeratin expression but shows positivity for high-molecular-weight cytokeratins. Consequently, staining for High-Molecular-Weight cytokeratins (HMW) was performed using the Ventana CONFIRM anti-keratin antibody, while Low-Molecular-Weight cytokeratins (LMW) were assessed with Ventana anti-CK8/18 and Ventana anti-CK7 antibodies.

In some cases, additional staining with the Ventana anti-p40 (BC28) antibody was carried out; p40 protein is expressed in epithelial tissues, and its immunoreactivity is typically positive in squamous cell carcinomas.

To determine HPV involvement, immunohistochemical staining with the Ventana CINtec® anti-p16INK4a antibody was performed. This antibody targets the p16 antigen, which is normally expressed at low levels in terminally differentiated cells and is undetectable by IHC. HPV infection and expression of the viral oncoprotein E7 lead to p16 overexpression; thus, p16 positivity serves as a surrogate marker of HPV infection, which is associated with the majority of anal squamous cell carcinoma cases.

For differential diagnosis with urothelial neoplasms, immunohistochemical staining with the Ventana GATA3 antibody was used. The GATA3 antigen is involved in the development and differentiation of the urothelium, the epithelial lining of the urinary bladder, and parts of the urinary tract. Its expression in urothelial cells makes it a valuable diagnostic marker for urothelial carcinoma.

Results

In this study, cases received by the Pathological Anatomy Department of the S. Rita Clinic between April 2022 and February 2025 were extracted using data-archive software. Using the topographical search criteria “ANUS, NAS”, 475 cases of anal pathologies were extracted, of which only 13 cases, including one recurrent case, were reported as squamous cell carcinoma.

The reported cases of squamous cell carcinoma are listed in Table 4.

From Figure 1, it can be deduced that, despite the high incidence of anal region pathologies, the number of squamous cell carcinoma cases is very low, accounting for only 3% of reported anal region pathologies, thereby indicating that this pathology is rare.

Subsequently, an analysis was carried out on the incidence of the neoplasm in both sexes. Of the 13 cases reported, only 3 samples

belonged to males, highlighting that this disease has a higher incidence in the female population.

An analysis was performed on the incidence of neoplasia in the following age groups: age <20 years, age between 20-40 years, age between 40-50 years, age between 50-60 years, and age >65 years.

Figure 2 shows that the development and diagnosis of anal cancer are more common in older age groups; no cases of anal cancer were found in the under-20 age group or in the 20-40 age group, only two reported cases belong to the 40-50 age group, while most cases belong to the 50-60 age group (6 cases) and the over-65 age group (5 cases, one of which is a recurrence). It can be inferred that there is an increase in the incidence of this disease from the age of 50 onwards.

In addition to gender and age, one of the factors with the greatest impact on the incidence of this disease is the presence of HPV infection, which can be identified through immunohistochemical testing for the detection of the p16 protein. Almost all of the samples analysed and reported were positive for this protein, indicating the presence of HPV infection.

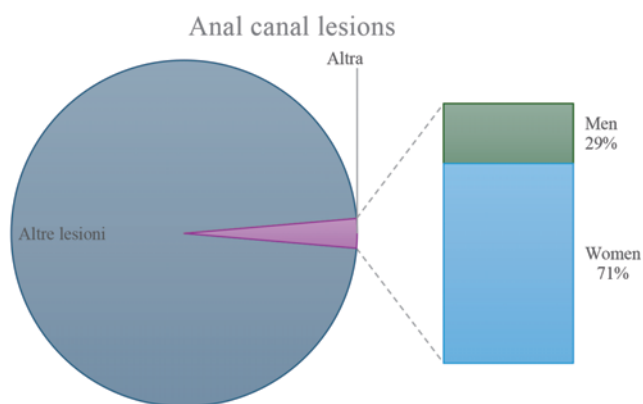


Figure 1. Incidence of squamous cell carcinoma of the anus.

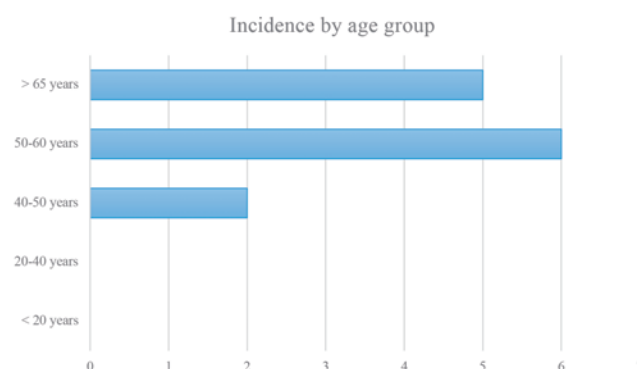


Figure 2. Incidence of squamous cell carcinoma of the anus in different age groups.

Table 4. Diagnosis of squamous cell carcinoma reported between April 2022 and February 2025 in the anatomic pathology laboratory of the S. Rita Clinic located in Vercelli.

N° of protocol	Age at diagnosis	Sex	Diagnosis
3637/22	79	F	Large cell nonkeratinizing squamous cell carcinoma
744/23	62	F	Differentiated squamous cell carcinoma
840/23-a	70	F	Squamous cell carcinoma with basaloid and keratinizing features
921/23	80	F	Basaloid squamous cell carcinoma
4386/23	83	M	Basaloid squamous carcinoma poorly differentiated
4599/23	70	F	Poorly differentiated nonkeratinizing squamous cell carcinoma
5495/23 (Recurrence 3637/22)	80	F	Nonkeratinizing squamous cell carcinoma
1062/24	52	M	Keratinizing squamous cell carcinoma
1595/24	77	F	Keratinizing squamous cell carcinoma
1906/24	47	F	Non-keratinizing squamous cell carcinoma with basaloid features
3404/24-a	65	M	Moderately differentiated keratinizing squamous cell carcinoma
3593/24	52	F	Keratinizing squamous cell carcinoma
4984/24-a	53	F	Squamous cell carcinoma
644/25	65	F	Moderately differentiated keratinizing squamous cell carcinoma

Examples of the cases analysed are given below. Case number 921/23 belongs to a female patient and shows the presence of a squamous cell carcinoma of the basaloid variety (Figure 3), ulcerated, focally infiltrating the hypodermis and bundles of striated muscle tissue with perineural infiltration (grade of differentiation G3 and stage pT2). The sample is positive for immunohistochemical staining for the detection of cytokeratins 5/6 (Figure 4a) and p40 protein (Figure 4c), while it is focally positive for staining for the detection of cytokeratins 8/18 (Figure 4b) and p16 protein. Case number 3637/22 belongs to a female patient and shows a large cell squamous carcinoma (Figure 5), non-keratinising, infiltrating (stage pT2), which tested positive for immunohistochemical staining for the detection of p16 protein. The patient had a recurrence of the disease the following year, in 2023. The recurrence, with protocol number 5495/23, shows fragments of non-keratinising squamous cell carcinoma (Figure 6a) that tested positive for immunohistochemical staining for the detection of cytokeratin 5/6 expression (Figure 6b), p16 protein (Figure 6c) and p40 protein. Case number 1062/24 belongs to a male patient and shows the presence of an exophytic keratinising squamous cell carcinoma in the context of condyloma (Figure 7) without ulceration, infiltrating the papillary dermis but without neural or vascular infiltration (differentiation grade G1 and stage pT1). The lesion is positive for immunohistochemical staining that detects overexpression of the p16 protein.

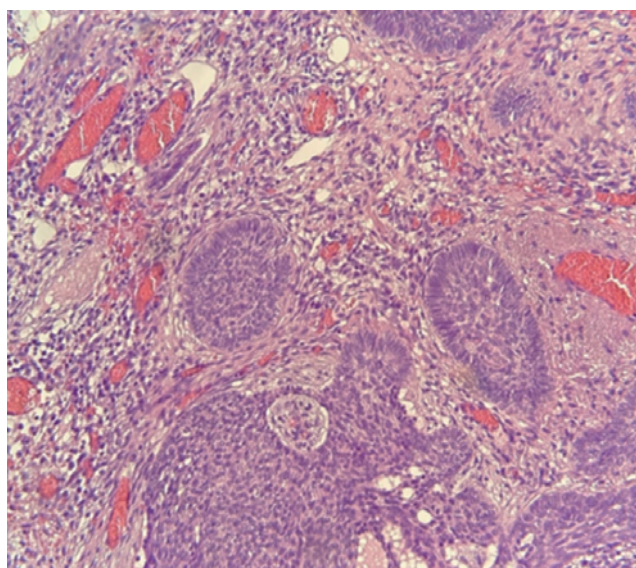


Figure 3. Haematoxylin and eosin staining of a slide showing Squamous Cell Carcinoma of the Anus (SCCA) 921/23 showing basaloid features (20x magnification).

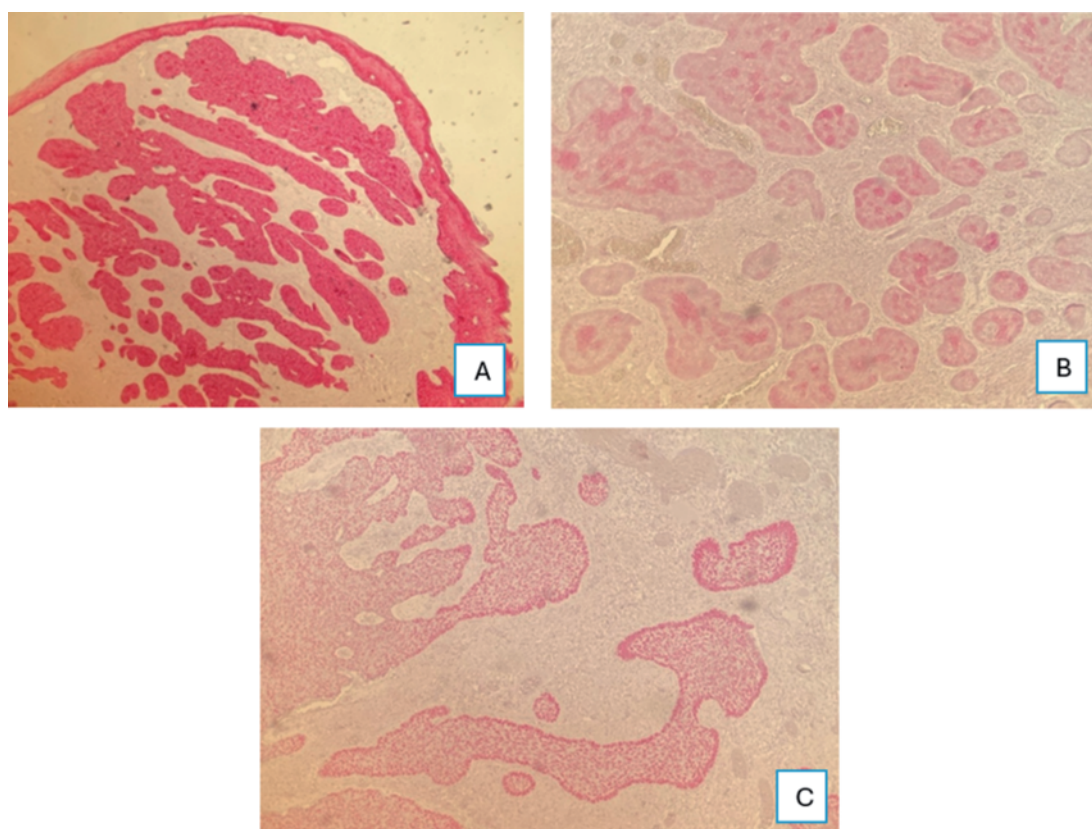


Figure 4. A) Squamous Cell Carcinoma of the Anus (SCCA) 921/23 sample positive for Ventana Anti-CK5/6 immunohistochemical staining (5x magnification); B) SCCA 921/23 sample with focal positivity for Ventana Anti-CK8/18 immunohistochemical staining (10x magnification); C) SCCA 921/23 sample positive for Ventana Anti-p40 immunohistochemical staining (10x magnification).

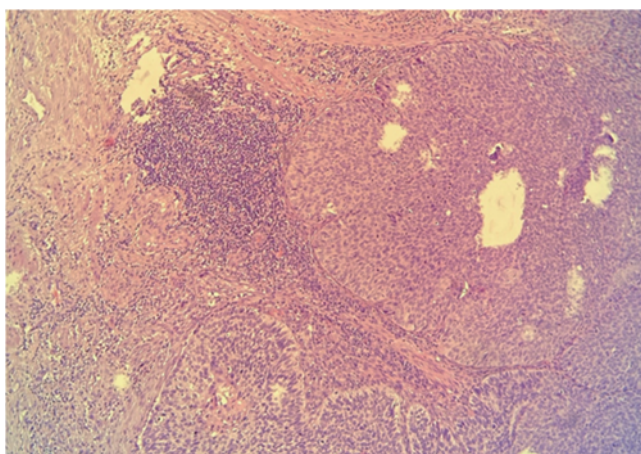


Figure 5. Haematoxylin and eosin staining of a slide showing Squamous Cell Carcinoma of the Anus (SCCA) 3637/22 (10x magnification).

Discussion

Following the collection and evaluation of the data obtained, various histopathological and epidemiological considerations were made. An initial analysis of the data showed that squamous cell carcinoma of the anus is a rare disease, as it was found in only 3% of biopsies of the anal region, and that it has a higher incidence in females and individuals over the age of 50, a result that is consistent with studies in the literature. A recent study conducted in 2023 by Mignozzi *et al.* provides an overview of the incidence and mortality of anal cancer up to 2020 in European countries and some countries around the world. This study shows that in most of the countries considered, the incidence of this neoplasm is higher in the female population.¹⁵ Subsequently, morphological analysis showed that the least common subtype was large non-keratinising cells, with the others being found in higher percentages. However, a sample with squamous cell carcinoma of the anus may present multiple aspects of multiple subtypes and may be subject to inter-observer variability. Therefore, the study by Fenger *et al.* showed that the subdivision into subtypes is not relevant for the management and treatment of the disease, therefore, focusing attention on the incidence of the various subtypes from a diagnostic point of view is unnecessary.^{9,16}

Immunohistochemical analysis has shown that this neoplasm

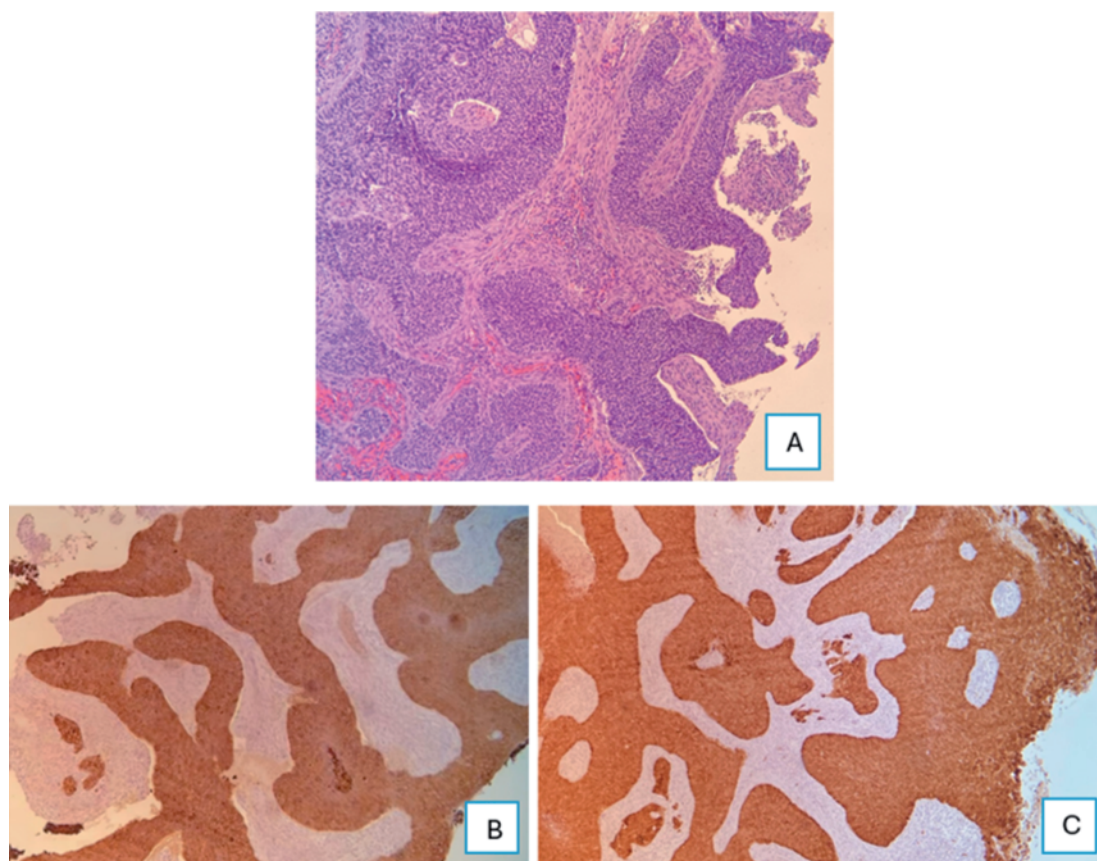


Figure 6. A) Haematoxylin and eosin staining of a slide showing Squamous Cell Carcinoma of the Anus (SCCA) 5495/23 (recurrence 3637/22) (10x magnification); B) SCCA 5495/23 sample positive for Ventana Anti-CK5/6 immunohistochemical staining (10x magnification); C) SCCA 5495/23 sample positive for Ventana anti-p16INK4a immunohistochemical staining (10x magnification).

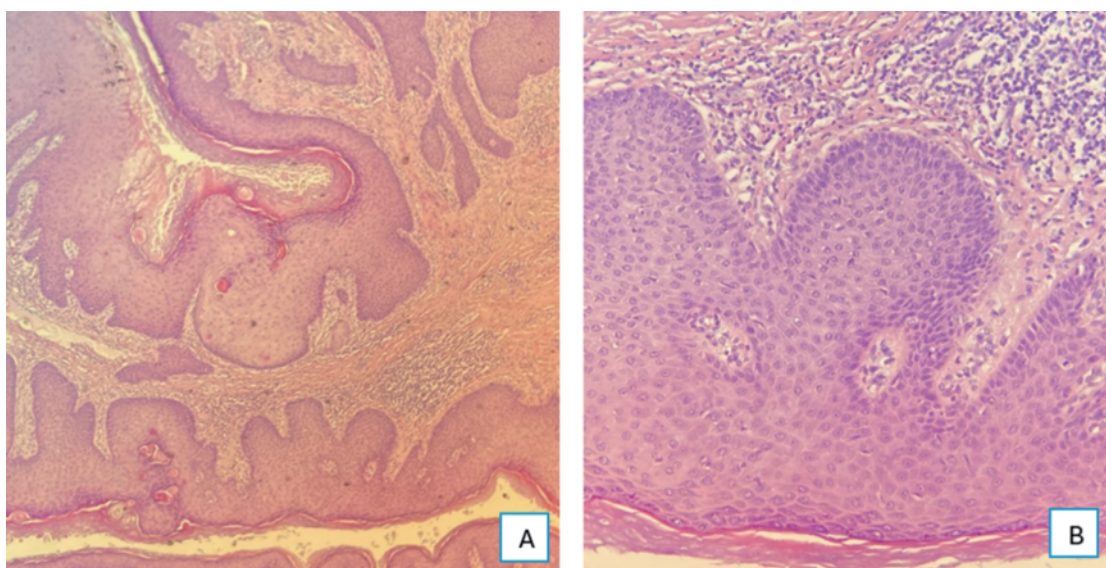


Figure 7. Haematoxylin and eosin staining of a slide showing Squamous Cell Carcinoma of the Anus (SCCA) 1062/24; A) 5x magnification; B) 20x magnification.

often tests positive for the p16 protein, indicating the presence of HPV viral infection as a carcinogen. More than 80% of anal cancer cases are, in fact, associated with HPV infection.^{17,18} In a study of 386 cases of squamous cell carcinoma of the anus, 90% of women and 63% of men diagnosed with invasive squamous cell carcinoma of the anal region were found to be infected with HPV.¹⁹

HPV is an important factor in anal carcinogenesis. The production of the viral oncoproteins E6 and E7 by the virus influences various processes such as angiogenesis, DNA repair, growth arrest, and apoptosis. The E6 protein associates with E6AP and subsequently binds to p53, promoting its degradation through the ubiquitination process. As a result, the function of p53 as a tumour suppressor is compromised. On the other hand, the E7 protein causes the degradation of pRb, which normally acts as a tumour suppressor.^{5,20} The loss of function of p53 and pRb, induced by HPV, not only promotes uncontrolled cell proliferation, but also instability, leading to an accumulation of mutations and thus to the possible development of neoplasms,⁶ including SCCA. Future developments could focus on a greater understanding of these molecular mechanisms and the development of immunotherapy based on the inhibition of E6 and E7.

HPV infection is often asymptomatic and subclinical; studies using PCR techniques to detect HPV DNA have shown that up to 90% of HPV infections are subclinical or latent.²¹ This suggests the importance of preventing squamous cell carcinoma of the anal region; this has also been highlighted by the ANCHOR and SPANC studies, which investigated the potential benefits of preventing this neoplasm.^{22,23}

In order to make a correct differential diagnosis of lower gastrointestinal tract neoplasms, the following immunohistochemical tests were performed on some samples: neuroendocrine markers, including synaptophysin and chromogranin, which are usually positive in Neuroendocrine Tumours (NETs) of the gastrointestinal tract; search for GATA3, which identifies urothelial cells and is therefore expressed in urothelial carcinoma; search for p40, which is present in SCCA but not in adenocarcinoma. Immunohistochemistry meth-

ods are important for a correct diagnosis, as the anal canal can be subject to many different types of neoplasms, including adenocarcinoma, neuroendocrine tumours, melanomas, lymphomas or other mesenchymal tumours.²⁴

Conclusions

Squamous cell carcinoma of the anal region is an uncommon but increasingly prevalent disease that encompasses a range of histomorphological characteristics and, for this reason, can be misdiagnosed. Therefore, anamnestic and clinical studies are essential for a correct understanding and differential diagnosis.

Given the crucial importance of HPV as a risk factor for the development of SCCA, the introduction of an HPV vaccination programme in high-risk populations could significantly reduce the incidence of squamous cell carcinoma of the anus, as already suggested in previous studies.

Currently, there are no biomarkers capable of predicting the response to a specific clinical treatment for this disease,²⁴ so future developments could include a more extensive molecular study of anal squamous cell carcinoma and the identification of proteins that can be used as checkpoints in immunotherapy or targeted therapy.

In the future, it would also be interesting to evaluate the association of this neoplasm with the presence of certain Human Leucocyte Antigens (HLAs) alleles. Since most anal canal carcinomas are associated with HPV viral infection, the development of the neoplasm could be associated with the presence of specific HLAs.¹ Currently, there are no reports of studies that have addressed this association, and attempts to identify other markers of susceptibility to developing the disease have been unsuccessful.

Certainly, expanding the case series, perhaps through collaboration between multiple hospitals, could be useful in the diagnostic, prognostic, and predictive management of response to therapy in patients with anal squamous cell carcinoma.

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Availability of data and materials: all data generated or analyzed during this study are included in this published article.

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