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Pulmonary edema in acute opioid intoxication: the role of AQP1

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ABSTRACT

Pulmonary edema is the most frequently observed macroscopic finding in autopsies of acute opioid intoxication. However, its underlying pathogenic mechanisms remain unclear and are still under debate. Some opioids can directly modulate aquaporins (AQPs), membrane proteins that regulate intra- and extracellular water flux and play a key role in maintaining osmotic homeostasis.

This study aims to investigate, in a forensic case series of acute opioid intoxication deaths (heroin, morphine, and methadone), the potential role of pulmonary endothelial AQP1 expression in the development of pulmonary edema.

Fourteen deaths due to opioid intoxication and fourteen control subjects who died from violent or natural causes without opioid intake were examined; among controls, seven deaths were related to neurogenic shock and seven to cardiogenic shock.

Our results showed an overall inverse relationship between pulmonary edema severity and AQP1 expression, with lower AQP1 levels corresponding to greater edema severity. However, compared with controls, subjects who died from acute opioid intoxication tended to maintain relatively higher pulmonary AQP1 expression across increasing degrees of edema severity. In fact, the data analysis revealed that only with severe pulmonary edema (grades 3 or 4, based on hematoxylin-eosin staining) was AQP1 expression significantly higher in subjects who died from acute opioid intoxication compared to controls.

This modulation of AQP1 in severe opioid-related pulmonary edema may have important clinical implications for understanding disease progression and mechanisms of pulmonary pathology exacerbation.

1. Introduction

In recent years, it is estimated that of the approximately 600,000 global deaths attributable to substance use, 25% are related to acute opioid intoxication [1]. The term “opioids” refers to a class of alkaloids derived from opium, either naturally occurring (morphine, heroin) or semi-synthetic (methadone), which primarily act as pure agonists of μ- and κ-receptors in the central nervous system [2–6].

Pulmonary edema is the most frequently observed macroscopic finding in autopsies of acute opioid intoxication, with reported incidence ranging from 48% to 85% [7–10]. The underlying pathogenic mechanisms remain unclear and are still debated [7,11–13]. The anaphylactic hypothesis, based on massive histamine release, lacks experimental support because no hemodynamic alterations or increases in pulmonary IgE levels have been demonstrated [7,14]. Similarly, the cardiogenic hypothesis has been discounted owing to the absence of hemodynamic changes [10]. The neurogenic hypothesis, secondary to depression of the respiratory centers, was also excluded because comparable levels of hypoxia have been reported in barbiturate overdoses without pulmonary edema [13–17]. More recently, however, evidence has favored a mechanism involving increased alveolar–capillary membrane permeability, leading to translocation of water and proteins into the alveolar space [10]. In opioid intoxication, the protein content of alveolar fluid is substantially higher than that observed in cardiogenic

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edema, further supporting a permeability-type edema [10,18]. Nonetheless, histological confirmation of a direct toxic injury to the alveolar–capillary membrane is still lacking [7]. Consequently, the prevailing view is that pulmonary edema related to acute opioid intoxication is primarily associated with increased alveolar–capillary permeability, although the specific pathophysiological mechanism has not yet been conclusively identified.

Aquaporins (AQPs), membrane proteins regulating intra- and extracellular water flux and essential for osmotic homeostasis, are emerging as important mediators of tissue edema [19,20]. In the lungs, AQP1, expressed in alveolar capillary endothelium, and AQP5, localized to type I alveolar epithelial cells, are implicated in osmotic transport and may play a role in the development and resolution of non-cardiogenic edema [21–24].

In the literature, particularly regarding the possible interaction between opioids and AQPs, experimental mouse models have shown that morphine administration is associated with downregulation of cerebral AQP1 [25,26], whereas an upregulation of AQP3 in the colon has been observed in relation to other AQPs [27]. Furthermore, in another recent study conducted on murine models, the semi-synthetic opioid oxycodone induces an up-regulation of pulmonary AQP1 and AQP5 [28].

Therefore, with regard to the specific regulation of AQP1 by opioids, the current literature reports that, in mouse models, oxycodone is associated with upregulation of AQP1 in the lungs, whereas morphine administration is associated with downregulation of AQP1 in the brain. To date, no studies have specifically investigated human tissues to evaluate the interaction between opioids and pulmonary AQP1.

AQPs have also garnered forensic interest, particularly in differentiating freshwater from saltwater drowning, based on immunohistochemical analysis of renal and pulmonary AQP2, cerebral AQP1 and AQP4, and pulmonary AQP1 and AQP5 [29–35]. In the field of forensic toxicology, only one study has investigated the role of AQPs in cases of acute intoxication. Specifically, the study by Wang et al. examined the neurotoxicity of methamphetamine, hypothesizing that it may modify the structure of the blood-brain barrier. The study reported an increased expression of AQP4, suggesting that methamphetamine use compromises the integrity of the blood-brain barrier by altering tight junctions and the extracellular matrix composition [36].

Despite these insights, the pathophysiology of opioid-induced pulmonary edema remains incompletely understood, with increased alveolar-capillary permeability considered the most plausible mechanism. However, the specific trigger for this phenomenon remains unidentified.

The present study aims to investigate, in a forensic case series of acute opioid intoxication deaths (heroin, morphine, and methadone), the potential role of pulmonary endothelial AQP1 expression in the development of pulmonary edema.

2. Materials and methods

A retrospective observational study was conducted on the autopsy series of the Institute of Legal Medicine of Canton Ticino (Switzerland) and the Institute of Legal Medicine of Bologna (Italy), covering the period from 2020 to 2024.

Deaths were included when histological examination showed pulmonary edema due to: (a) acute opioid intoxication (cases); (b) violent or natural causes with toxicological analyses negative for opioids (controls). In subjects with detectable levels of other illicit drugs and/or medications in the blood, inclusion in the study was allowed only when the concentrations of these substances were not considered sufficient to account for the cause of death; therefore, only cases in which such concentrations fell within the therapeutic range, without reaching toxic or lethal levels, were included. Cases with evident putrefactive changes were excluded to avoid interpretative bias related to tissue degradation of AQP, with a maximum postmortem interval of 72 h between the presumed time of death and autopsy with sampling being considered. Fourteen deaths due to opioid intoxication and 14 control subjects who died from violent or natural causes without opioid intake were selected.

For each case and control, five samples were taken from each pulmonary lobe and subjected to histological and immunohistochemical analyses.

Samples were fixed in formalin, dehydrated in alcohol, and embedded in paraffin. Sections were then cut and processed for hematoxylin-eosin (HE) staining and immunohistochemical (IHC) reactions. The anti-aquaporin-1 antibody (AB2219, Merck Millipore), a polyclonal rabbit antibody, was used in this study. The antibody was diluted 1:50 using Bond Antibody Diluent and incubated for 15 min at 25 °C. For antigen retrieval, sections were treated with Leica Bond Epitope Retrieval Solution 2 and incubated for 30 min at 100 °C using the Leica Bond III system.

Each hematoxylin–eosin-stained slide was independently and blindly evaluated by the same two investigators for the degree of pulmonary edema. The pulmonary edema was assessed as the extension of the filling of alveolar space with proteinaceous fluid and debris. Although there is no current consensus for evaluation of pulmonary edema, we combined two previoulsy suggested pathological methods [37,38]. The semiquantitative 1–4 grading scale roughly considered the extension area of the edema on each slide (1 = mild, 1%–20%, 2 = moderate, 21%–40%; 3 = marked, 41%–80%; 4 = severe, 81%–100%). Similarly, each immunohistochemically stained slide was evaluated, using the same semiquantitative scoring system, for the intensity of AQP1 expression in the pulmonary endothelium, where AQP1 is constitutively expressed in microvascular endothelial cells across the pulmonary capillary network, including peribronchial vessels and the visceral pleura [39]. Thus, the reactions were classified as mild (grading 1), moderate (grading 2), marked (grading 3), and severe (grading 4) [40].

Descriptive results were summarized using cross-tabulations of AQP1 expression scores by pulmonary edema grade.

Because multiple samples and two blinded assessments were obtained for each subject, observations were not statistically independent. To account for the clustered structure of the data, regression analyses were performed using subject-level cluster resampling.

Associations between AQP1 expression and edema severity, case-control status, and investigator were evaluated using linear regression models with cluster-robust variance estimation. Confidence intervals and p-values were obtained via nonparametric bootstrap with 10,000 replications, resampling subjects as the unit of analysis. Bias-corrected and accelerated (BCa) confidence intervals were computed to improve coverage accuracy and to account for potential bias, skewness, and ties in the bootstrap distribution [41].

An interaction term between edema severity and group was explored in secondary analyses to assess whether the strength of the edema-AQP1 association differed between cases and controls.

All analyses were conducted using Stata 18 (StataCorp. 2023. *Stata Statistical Software: Release 18.* College Station, TX: StataCorp LLC). Statistical significance was set at $\alpha = 0.05$ (two-sided).

3. Results

The epidemiological, forensic, toxicological, and histological characteristics of cases and controls are summarized in Table 1. In cases of acute opioid intoxication, the most frequently detected substance was methadone alone (7 out of 14 cases, 50%), whereas in two cases it was co-administered with morphine; finally, deaths related to the sole intake of morphine accounted for 5 out of 14 cases (35.71%). Opioid concentrations were consistently higher than the values considered as fatal blood-plasma concentrations, corresponding to levels equal to or greater than 50 μg/L-1000 μg/L for methadone and 100 μg/L for morphine. Regarding the cause of death in the control group, half of the subjects died from neurogenic shock, while the remaining half died from cardiogenic shock. The manner of death was determined based on the circumstantial data of each case, with particular attention to

Table 1
Autopsy, toxicological, and histological characteristics of the cases and controls included in the study.

		Autopsy and toxicological findings					Histological findings	
		Age	**Sex**	**Cause of death**	**Other blood toxicity findings (at therapeutic levels, neither toxic nor lethal)**	**Manner of death**	**Pulmonary edema (HE) grading** Carried out on 5 pulmonary areas	**AQP1 expression grading** Carried out on 5 pulmonary areas
Cases	*n 1*	62	Male	Methadone intoxication (Methadone concentration 80 μg/L)	Benzodiazepines, loperamide, ethanol	Accident	Investigator 1: 2, 3, 3, 2, 2 Investigator 2: 2, 3, 3, 2, 3	Investigator 1: 4, 3, 3, 4, 4 Investigator 2: 4, 3,2, 4, 4
	n 2	55	Female	Methadone intoxication (Methadone concentration 470 μg/L)	Cocaine, amisulpride, difenidramina, amitriptilina, losartan, rivaroxiban, benzodiazepines	Accident	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 3, 3, 3, 3, 3 Investigator 2: 3, 3, 3, 3, 3
	n 3	47	Female	Methadone intoxication (Methadone concentration 1600 μg/L)	Benzodiazepines, ethanol	Suicide	Investigator 1: 2, 3, 3, 2, 4 Investigator 2: 2, 3, 3, 2, 3	Investigator 1: 4, 3, 3, 4, 3 Investigator 2: 4, 3, 3, 4, 2
	n 4	32	Male	Methadone and morphine intoxication (Methadone concentration 70 μg/L; Morphine concentration 110 μg/L)	Morphine, levopromazina, quetiapina, zuclopentixolo, cetirizina, idrossizina, biperidene, pregabalin, ethanol, benzodiazepines	Accident	Investigator 1: 2, 2, 4, 3, 4 Investigator 2: 2, 2, 4, 3, 4	Investigator 1: 4, 4, 3, 4, 3 Investigator 2: 4, 4, 4, 4, 3
	n 5	23	Female	Methadone intoxication (Methadone concentration 460 μg/L)	THC, ketamina, ethanol, benzodiazepines	Suicide	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 3, 3, 3, 3, 3 Investigator 2: 3, 3, 3, 3, 3
	n 6	23	Male	Methadone intoxication (Methadone concentration 420 μg/L)	Cocaine, THC, benzodiazepines	Accident	Investigator 1: 2, 2, 2, 2, 3 Investigator 2: 2, 2, 2, 2, 3	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 3
	n 7	33	Male	Methadone intoxication (Methadone concentration 1600 μg/L)	Cocaine, aripipazolo, pregabalin, benzodiazepines	Accident	Investigator 1: 2, 2, 3, 4, 3 Investigator 2: 2, 2, 2, 4, 3	Investigator 1: 4, 4, 3, 3, 3 Investigator 2: 4, 4, 4, 3, 3
	n 8	58	Male	Methadone intoxication (Methadone concentration 1600 μg/L)	Cocaine, THC, ethanol, benzodiazepines	Accident	Investigator 1: 2, 2, 2, 2, 2 Investigator 2: 2, 2, 2, 2, 2	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4
	n 9	57	Male	Morphine intoxication (Morphine concentration 6900 μg/L)	Meperidine	Accident	Investigator 1: 4, 4, 3, 3, 4 Investigator 2: 3, 4, 3, 3, 4	Investigator 1: 3, 3, 2, 3, 2 Investigator 2: 3, 2, 2, 3, 2
	n 10	39	Female	Methadone intoxication (Morphine concentration 682 μg/L)	Alprazolam, delorazepam	Accident	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 3, 3, 3, 2, 3 Investigator 2: 3, 3, 2, 3, 2
	n 11	57	Male	Methadone intoxication (Morphine concentration 1050 μg/L)	/	Accident	Investigator 1: 3, 4, 4, 4, 2 Investigator 2: 3, 4, 4, 4, 3	Investigator 1: 2, 3, 2, 2, 3 Investigator 2: 2, 3, 2, 3, 3
	n 12	46	Male	Methadone intoxication (Morphine concentration 320 μg/L)	Diazepam, nordiazepam, mirtazapine	Accident	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 3, 3, 2, 3, 2 Investigator 2: 3, 2, 3, 3, 2
	n 13	45	Male	Methadone and morphine intoxication (Methadone concentration 100 μg/L Morphine concentration 1400 μg/L)	D/levomethorphan, D/levorfan	Accident	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4 4, 4	Investigator 1: 2, 2, 3, 3, 3 Investigator 2: 2, 3, 3, 2, 2
	n 14	31	Male	Morphine intoxication (Morphine concentration 400 μg/L)	Cocaine, ethanol	Accident	Investigator 1: 4, 4, 4, 3, 2 Investigator 2: 4, 4, 4, 3, 3	Investigator 1: 2, 3, 3, 2, 3 Investigator 2: 2, 3, 3, 2, 2
Controls	*n 1*	75	Male	Neurogenic shock (gunshot)	/	Suicide	Investigator 1: 1, 1, 2, 2, 1 Investigator 2: 1, 1, 1, 2, 1	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4
	n 2	92	Male	Neurogenic shock (gunshot)	Tradozone, amlodipina	Suicide	Investigator 1: 1, 1, 1, 1, 1 Investigator 2: 1, 1, 1, 1, 1	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4
	n 3	15	Male	Neurogenic shock (head trauma)	Valproato, pregabalin, quetiapina, ciprofloxacina	Accident	Investigator 1: 3, 3, 3, 3, 3	Investigator 1: 3, 2, 2, 1, 3

(*continued on next page*)

Table 1 (*continued*)

	Autopsy and toxicological findings					Histological findings	
	Age	**Sex**	**Cause of death**	**Other blood toxicity findings (at therapeutic levels, neither toxic nor lethal)**	**Manner of death**	**Pulmonary edema (HE) grading** Carried out on 5 pulmonary areas	**AQP1 expression grading** Carried out on 5 pulmonary areas
n 4	56	Male	Haemorrhagic shock	/	Accident	Investigator 2: 3, 3, 3, 3, 2 Investigator 1: 1, 1, 2, 1, 1 Investigator 2: 1, 1, 1, 1, 1	Investigator 2: 3, 3, 3, 2, 3 Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4
n 5	78	Male	Neurogenic shock (head trauma)	Ketamina	Accident	Investigator 1: 1, 1, 1, 1, 1 Investigator 2: 1, 1, 1, 1, 1	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4
n 6	38	Male	Cardiac tamponade (aorta dissection)	/	Natural	Investigator 1: 3, 4, 3, 4, 3 Investigator 2: 4, 4, 3, 4, 4	Investigator 1: 1, 2, 1, 2, 1 Investigator 2: 1, 1, 1, 2, 1
n 7	19	Male	Neurogenic shock (head trauma)	Ketamina, benzodiazepines	Accident	Investigator 1: 3, 3, 3, 3, 4 Investigator 2: 3, 3, 3, 4, 4	Investigator 1: 1, 1, 1, 2, 1 Investigator 2: 1, 1, 1, 1, 1
n 8	77	Male	Cardiac tamponade (right ventricle rupture)	Tamsulosina	Natural	Investigator 1: 1, 1, 1, 1, 1 Investigator 2: 1, 1, 1, 1, 2	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 3
n 9	84	Male	Neurogenic shock (spinal trauma)	Tamsulosina	Accident	Investigator 1: 3, 4, 4, 4, 2 Investigator 2: 3, 4, 4, 4, 3	Investigator 1: 1, 1, 1, 1, 2 Investigator 2: 1, 2, 1, 1, 2
n 10	42	Male	Myocardial infarction	Clozapina, paliperidone, benzodiazepines	Natural	Investigator 1: 3, 3, 2, 3, 3 Investigator 2: 3, 3, 2, 3, 3	Investigator 1: 1, 2, 1, 1, 2 Investigator 2: 2, 2, 1, 2, 2
n 11	58	Female	Myocardial infarction	Citalopram	Natural	Investigator 1: 4, 4, 4, 4, 3 Investigator 2: 4, 4, 4, 4, 3	Investigator 1: 1, 2, 1, 1, 1 Investigator 2: 1, 2, 1, 1, 1
n 12	87	Male	Neurogenic shock (gunshot)	Paroxetine	Suicide	Investigator 1: 4, 4, 4, 2, 2 Investigator 2: 4, 4, 4, 2, 2	Investigator 1: 1, 2, 1, 2, 1 Investigator 2: 1, 1, 1, 1, 1
n 13	36	Female	Haemorrhagic shock	Ethanol	Accident	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 1, 1, 1, 1, 1 Investigator 2: 1, 1, 2, 1, 1
n 14	28	Male	Sudden cardiac death (arrhythmogenic right ventricular dysplasia)	/	Natural	Investigator 1: 4, 4, 4, 4, 4 Investigator 2: 4, 4, 4, 4, 4	Investigator 1: 2, 1, 1, 1, 2 Investigator 2: 1, 1, 1, 1, 2

Abbreviations: HE hematoxylin-eosin, AQP aquaporin-1.

distinguishing cases of acute intoxication in which suicidal intent had been explicitly declared from those in which the intoxication was deemed accidental. In the control group, deaths were associated with traumatic events of either suicidal origin (e.g., gunshot wounds) or accidental origin (e.g., head trauma in car crashes), as well as with natural events (e.g., cardiac tamponade due to aortic dissection). (See Figs. 1–4.)

Across all evaluated slides ($n = 280$), which were independently and blindly assessed by the same two investigators, AQP1 expression showed a clear inverse distribution with increasing pulmonary edema severity (Table 2). Low edema grades were predominantly associated with high AQP1 scores, whereas higher edema grades corresponded to progressively lower levels of protein expression. In particular, all slides graded as mild edema (grading 1) exhibited severe AQP1 expression, while severe edema (grading 4) was mostly associated with low or absent AQP1 staining.

To formally assess these relationships while accounting for the clustered structure of the data (multiple samples and investigators per subject), a cluster-bootstrapped linear regression model was fitted (Table 3). Edema severity was strongly and inversely associated with AQP1 expression: each one-point increase in edema score corresponded to an average decrease of 0.81 points in AQP1 expression (BCa 95% CI −0.91 to −0.68; $p < 0.001$). This means that higher pulmonary edema severity was consistently associated with lower AQP1 expression across all evaluated samples.

After adjustment for edema severity and investigator, cases showed significantly higher AQP1 expression compared with controls (b = +1.29; BCa 95% CI +1.02 to +1.56; p < 0.001). No systematic differences were observed between the two investigators (b = −0.01; 95% CI −0.09 to +0.07; $p = 0.787$), indicating good inter-observer consistency.

In group-specific analyses, the inverse association between edema severity and AQP1 expression was observed in both cases and controls but was stronger among controls (b = −0.93; BCa 95% CI −1.02 to −0.75) than among cases (b = −0.56; BCa 95% CI −0.68 to −0.40). The interaction between edema score and group, which contrasts these two slopes, was statistically significant (b = −0.56 − [−0.93] = +0.37; BCa

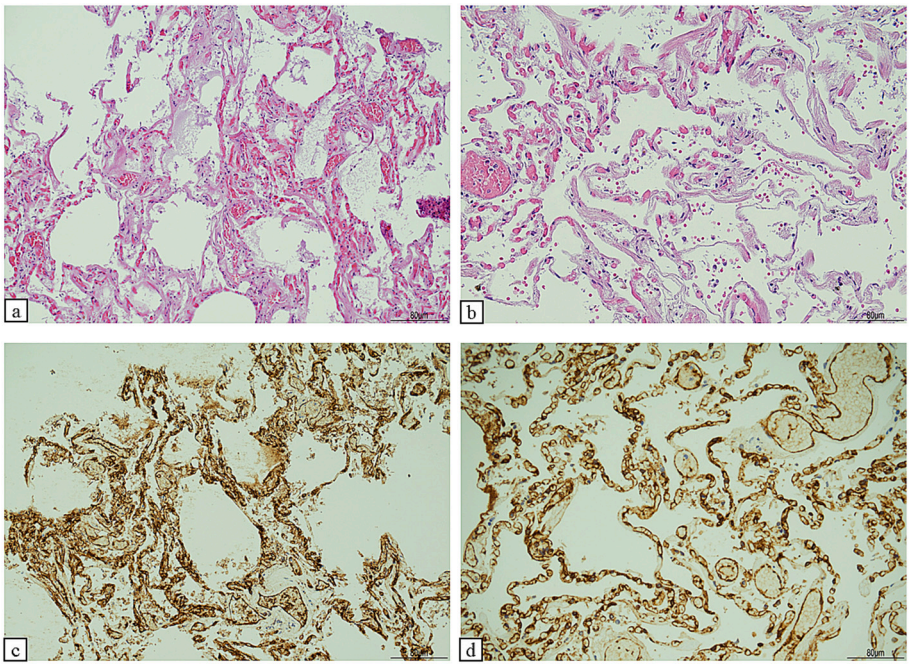

Fig. 1. Case group: histopathology of pulmonary edema at H&E stain grade 1 (enlargement: a 10×, b 20×), AQP expression grade 4 (enlargement: c 10×, d 20×). This refers specifically to case number 6 in Table 1.

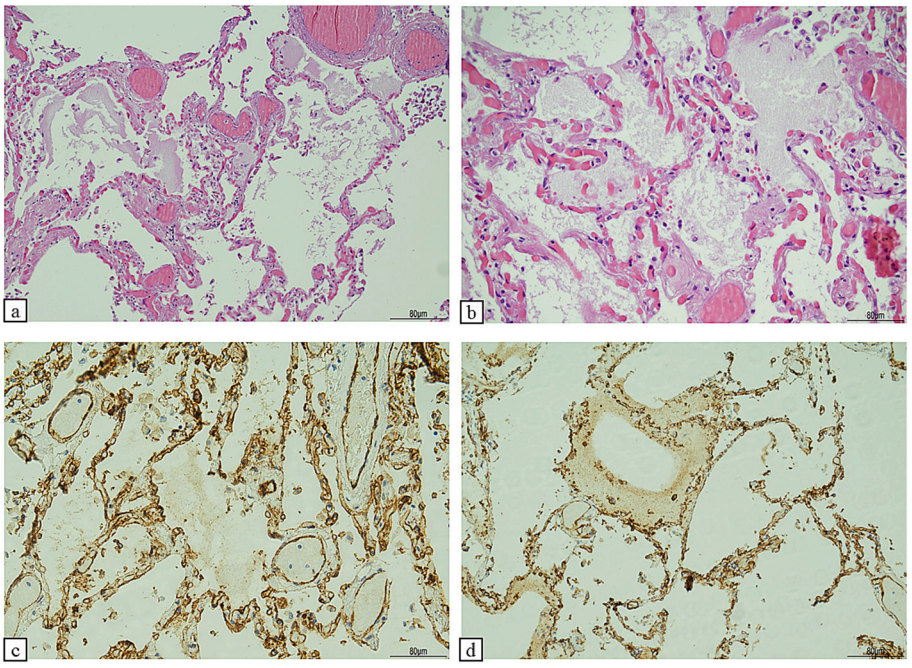

Fig. 2. Control group: histopathology of pulmonary edema at H&E stain grade 1 (enlargement: a 10×, b 20×), AQP expression grade 4 (enlargement: c 10×, d 20×). This refers specifically to case number 2 in Table 1.

95% CI +0.17 to +0.57; $p = 0.001$), indicating a weaker edema-AQP1 relationship in opioid intoxication cases. This means that, although AQP1 expression decreased with increasing edema severity in both groups, subjects who died from acute opioid intoxication tended to maintain relatively higher AQP1 expression even in the presence of severe pulmonary edema.

Taken together, these results suggest that, compared with controls, subjects who died from acute opioid intoxication tended to maintain relatively higher pulmonary AQP1 expression across increasing degrees of edema severity.

4. Discussion

Pulmonary edema in opioid intoxication remains incompletely understood, although current evidence supports increased alveolar-capillary membrane permeability as the prevailing hypothesis [7–10,14]. Experimental data suggest that pulmonary AQP1 may contribute to the pathophysiology of pulmonary oedema; however, its involvement in forensic cases of acute opioid intoxication has not yet been investigated [21–24]. Based on these findings, we hypothesized that pulmonary endothelial AQP1 expression may play a role in the

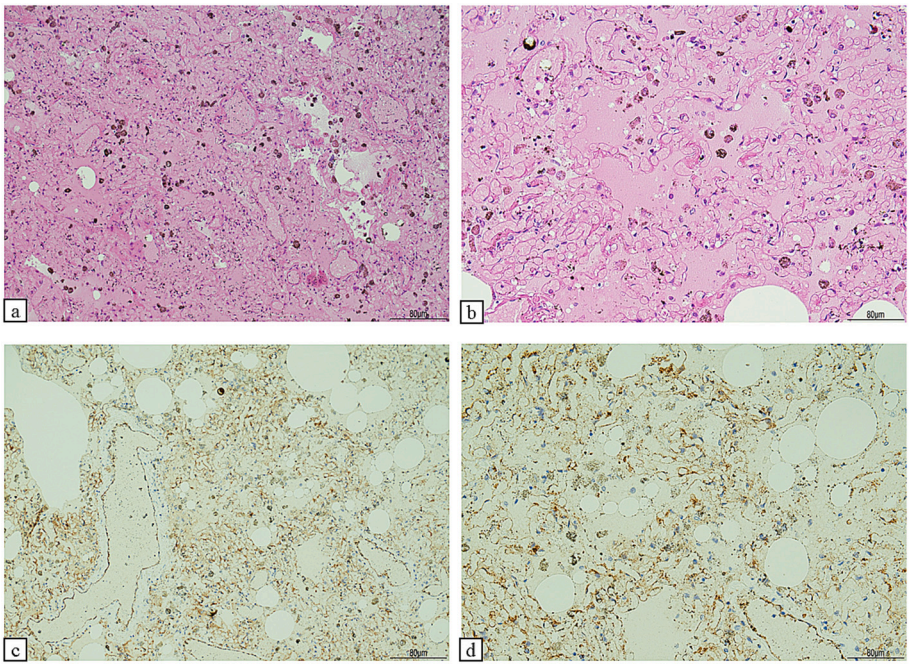

Fig. 3. Case group: histopathology of pulmonary edema at H&E stain grade 4 (enlargement: a 10×, b 20×), AQP expression grade 2 (enlargement: c 10×, d 20×). This refers specifically to case number 9 in Table 1.

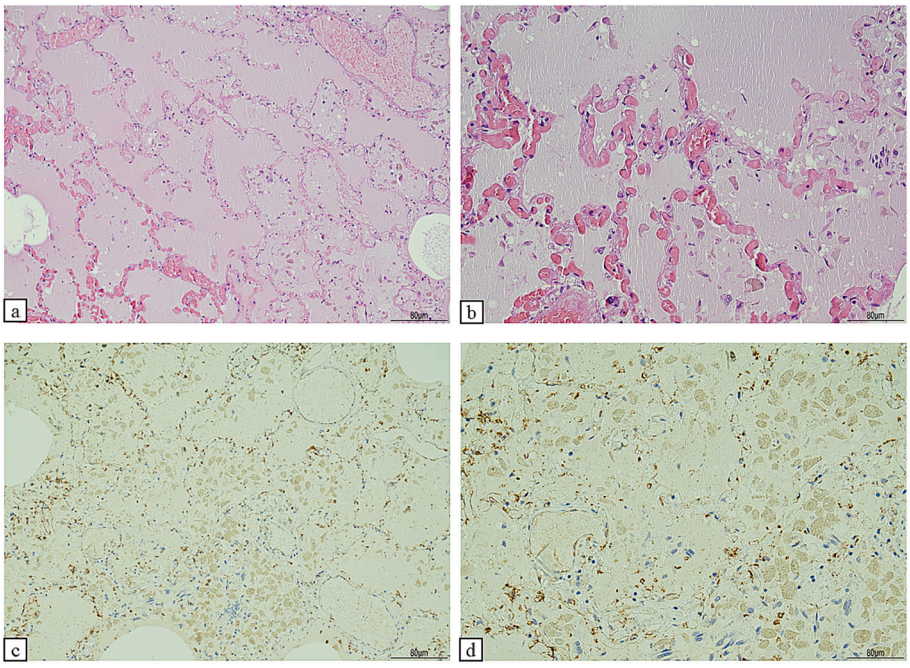

Fig. 4. Control group: histopathology of pulmonary edema at H&E stain grade 4 (enlargement: a 10×, b 20×), AQP expression grade 1 (enlargement: c 10×, d 20×). This refers specifically to case number 11 in Table 1.

development of pulmonary edema. The study was conducted on autopsy series from the Swiss Institute of Legal Medicine of Canton Ticino and the Italian Institute of Legal Medicine of Bologna, including 14 cases of acute opioid intoxication and 14 control deaths from violent or natural causes of neurogenic or cardiogenic shock, all with toxicology negative for opioids.

Comparison of the results of microscopic examination with hematoxylin-eosin staining and AQP1 immunohistochemistry showed that the severity of pulmonary edema was inversely proportional to AQP1 expression levels in all samples. Specifically, when pulmonary edema was mild on hematoxylin-eosin staining, AQP1 protein expression was higher, whereas increasing edema severity was associated with a progressive reduction of immunohistochemical positivity. This means that higher pulmonary edema severity was consistently associated with lower AQP1 expression across all evaluated samples. This overall pattern was observed in both cases and controls, suggesting that AQP1 modulation represents a common pathophysiological mechanism in the development of pulmonary edema, irrespective of the specific etiology. An inverse modulation of AQP1 expression was indeed observed with respect to edema severity, both in cases of pulmonary edema due to

Table 2
Cross-tabulation of AQP1 expression scores by pulmonary edema grade across all evaluated slides ($n = 280$). Values are n (% within edema column).

AQP1 expression	Pulmonary edema (HE)				Total ($n = 280$)
	Mild (1)	Moderate (2)	Marked (3)	Severe (4)	
	($n = 45$)	($n = 50$)	($n = 62$)	($n = 123$)	
Mild (1)	0 (0.0)	5 (10.0)	17 (27.4)	38 (30.9)	60 (21.4)
Moderate (2)	0 (0.0)	2 (4.0)	20 (32.3)	31 (25.2)	53 (18.9)
Marked (3)	0 (0.0)	4 (8.0)	21 (33.9)	53 (43.1)	78 (27.9)
Severe (4)	45 (100.0)	39 (78.0)	4 (6.5)	1 (0.8)	89 (31.8)

Abbreviations: HE hematoxylin-eosin, AQP1, aquaporin-1.

Table 3
Cluster-bootstrapped linear regression of AQP1 expression.

Predictor	Coefficient		*p*-value
	Estimate	BCa 95% CI	
Edema score (HE)	−0.81	−0.91, −0.68	<0.001
Case vs. control	+1.29	+1.02, +1.56	<0.001
Investigator 2 vs. 1	−0.01	−0.09, +0.07	0.787

Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval.

acute opioid intoxication and in control cases related to neurogenic and cardiogenic shock. However, in subjects who died from acute opioid intoxication with severe pulmonary edema, AQP1 expression was higher than in controls. Taken together, these findings suggest that, compared with controls, individuals who died from opioid intoxication exhibit relatively higher pulmonary AQP1 expression across increasing degrees of edema severity.

By comparing our results with those reported in the literature, it is primarily possible to confirm the hypothesis that AQP1 may play a role in the modulation of pulmonary edema [21–24]. Indeed, in both cases of opioid intoxication and in the control group comprising deaths unrelated to intoxication, AQP1 expression was consistently inversely correlated with the degree of edema. Specifically, AQP1 expression was higher when pulmonary edema was mild, whereas it was lower when pulmonary edema was severe.

However, a more detailed analysis of this general trend revealed that only when hematoxylin-eosin staining showed severe pulmonary edema (pulmonary edema intensity grades 3 or 4) AQP1 was expression significantly higher in subjects who died from acute opioid intoxication than in controls. Conversely, in cases of milder pulmonary edema (grades 1 and 2), this difference was not observed, and AQP1 expression levels were therefore similar between intoxication cases and controls.

Consequently, since this difference in AQP1 expression between intoxication cases and controls was observed only when pulmonary edema was more severe and not when it was mild, it can be hypothesized that opioids may induce AQP1 overexpression only in the more advanced stages of edema, when histological classification indicates greater edema severity.

This postulated modulation of AQP1 in severe opioid-related pulmonary edema could have pertinent clinical implications for disease progression and exacerbation of pulmonary pathology, consistent with reports of higher protein content in alveolar fluid during acute opioid intoxication compared with cardiogenic deaths [10,18]. It should be emphasised that the present study, focused on AQP1 expression, was not designed to investigate the aetiological mechanisms underlying the high concentration of proteinaceous material within the alveolar spaces, given that AQP1 exclusively regulates water transport and does not directly mediate solute movement. Nevertheless, the coexistence of increased AQP1 expression and elevated intra-alveolar protein levels in opioid intoxication cases could amplify pulmonary edema from a clinical standpoint, due to the rise in intra-alveolar osmotic pressure associated with AQP1 channel opening, which promotes additional water shifts from the vascular to the alveolar compartment.

Therefore, we hypothesize that opioids generally maintain AQP1 channels in an open state rather than acting exclusively in advanced stages of oedema. However, this effect is most evident in specimens from advanced pulmonary oedema. In control specimens without opioid exposure, AQP1 expression was inversely correlated with oedema severity: expression was higher in mild stages and progressively decreased as oedema worsened. By contrast, in cases of acute intoxication, AQP1 expression remained elevated despite increasing pulmonary oedema, suggesting that these substances may interact with AQP1 and stabilize the channel in an open conformation. This mechanism could facilitate continued osmotic water flux into the alveoli, driven by an osmotic gradient produced by hyperosmolar proteinaceous material in the alveolar space, thereby contributing to the development of acute pulmonary oedema.

Our study, however, has several limitations, the most relevant of which is the small number of cases. Although statistical significance was reached with respect to the edema-AQP1 relationship, the overall sample size was limited both by the restricted availability of forensic cases of acute opioid intoxication and by the requirement that autopsy sampling be performed within time frames adequate to minimize the risk of AQP protein degradation. Therefore, although the postmortem interval was standardized to a maximum of 72 h, the immunohistochemical expression of AQP1 may still reflect potential protein degradation; nonetheless, within the context of this study, the association between edema and AQP1 expression was found to be consistent in both cases and controls. In future studies, it would be of interest to investigate not only the immunohistochemical expression of AQP1 but also the corresponding gene expression at the mRNA level.

Furthermore, the marked heterogeneity of blood concentrations of morphine and methadone in subjects who died from acute opioid intoxication, together with the small sample size, precluded the possibility of performing statistical analyses to investigate correlations between specific opioid concentration ranges and distinct patterns of AQP1 expression and pulmonary edema.

A further limitation, as also shown in the accompanying tables, is the frequent co-intake of substances of abuse such as illicit drugs, alcohol, and medications in subjects who died from acute opioid intoxication and, in some instances, also among controls. Although inclusion in the study was permitted only when the concentration of the non-opioid substance was not considered sufficient to have caused death, co-consumption was present in all acute intoxication cases, with cocaine, alcohol, and benzodiazepines being most commonly represented. Among controls, three out of ten subjects did not use substances of abuse or medications, whereas in the remaining cases greater heterogeneity in the substances taken was observed. The lack of specific studies in the literature on the interaction between substances of abuse and in general, and in particular AQP1, the focus of our study, further complicates assessment of the impact of these factors. At present, indeed, the literature reports only a single forensic case series that analyzed changes in AQP expression in acute intoxication deaths, demonstrating an increased gene expression of AQP4 in the brain of subjects who died following methamphetamine use [35].

Regarding future research perspectives, and bearing in mind the aforementioned limitations inherent to forensic case series, it would first be advisable to replicate the present study in a larger cohort, including cases of opioid intoxication occurring in the absence of other substances or medications. In addition, control cases should consist of subjects with no history of drug or medication use, stratified according to the cause of death (for example, neurogenic or cardiogenic shock).

Moreover, it would be of great interest to further investigate the role

of other AQP isoforms expressed in the lung, particularly AQP5, in order to clarify whether it acts synergistically with or independently from AQP1 in modulating pulmonary water balance.

Another aspect to be considered concerns the potential clinical implications of these observations. If the modulation of AQP1 by opioids were to be confirmed, it could open the way to the development of targeted therapeutic strategies for the treatment of massive opioid-induced pulmonary edema, which underlies the acute asphyxia that increases the risk of death in these individuals, together with the consequences related to depression of the central respiratory centers. Specifically, by developing treatments that modulate AQP1 expression in cases of acute opioid intoxication, it may be possible to attenuate the severity of the edema and, consequently, improve both the clinical outcome and, potentially, the survival of these patients. In this regard, other authors have already hypothesized therapeutic approaches based on modulation of cerebral AQP4 for the management of ischemic edema [36] and colonic AQP3 for the treatment of morphine-induced constipation [27].

5. Conclusions

Our study demonstrated an inverse relationship between pulmonary edema severity and AQP1 expression across all samples where, in which AQP1 expression was inversely correlated with the degree of edema. However, compared with controls, subjects who died from acute opioid intoxication tended to maintain relatively higher pulmonary AQP1 expression across increasing degrees of edema severity. In fact, the data analysis revealed that only when hematoxylin-eosin staining showed severe pulmonary edema (grades 3 or 4) AQP1 was expression significantly higher in subjects who died from acute opioid intoxication than in controls.

Therefore, it can be hypothesized that opioids generally maintain AQP1 channels in an open state, rather than acting exclusively in the advanced stages of oedema. Nevertheless, this mechanism is most clearly observed in samples from advanced pulmonary oedema, where, in acute intoxication cases, AQP1 expression remained elevated despite increasing pulmonary oedema compared with controls, thereby supporting the hypothesis that these substances may interact with AQP1 and stabilize the channel in an open conformation. This could facilitate ongoing osmotic water movement into the alveoli, driven by an osmotic gradient generated by hyperosmolar proteinaceous material within the alveolar space, thereby contributing to the development of acute pulmonary oedema.

This AQP1 modulation in severe opioid-related pulmonary edema may carry relevant clinical implications for disease progression and pulmonary pathology exacerbation, aligning with reports of elevated alveolar fluid protein content in acute opioid intoxication versus cardiogenic deaths. Despite limitations due to small sample size and frequent polysubstance intake, our findings offer novel insights into the pathophysiological mechanisms of pulmonary edema in opioid intoxication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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